DIRECTIONS FOR A PRACTICAL COURSE IN CHEMICAL PHYSIOLOGY

W. CRAMER

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CHEMICAL PHYSIOLOGY

BY

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FOURTH EDITION

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. . . "Not that I want a due respect to other Mens Opinions; *but after all the greatest Reverence is due to Truth:* and I hope it will not be thought Arrogance to say, that perhaps we should make greater progress in the Discovery of rational and contemplative *knowledg*, if we *sought* it in the Fountain, *in the consideration of things themselves*, and made use rather of our own Thoughts than other Mens to find it: For I think we may as rationally hope to see with other Mens Eyes, as to know by other Mens Understandings. So much as we our selves consider and comprehend of Truth and Reason, so much we possess of real and true knowledg. The floating of other Mens Opinions in our Brains, makes us not one jot the more knowing, tho they happen to be true. What in them was Science, is in us but Opiniatrety; whilst we give up our Assent only to Reverend Names, and do not, as they did, imploy our own Reason to *understand* those *Truths* which gave them Reputation. . . . In the Sciences, every one has so much as he really knows and comprehends: What he believes only, and takes upon trust, are but shreds; which however well in the whole piece, make no considerable addition to his stock who gathers them. Such borrowed Wealth, like Fairy-mony, tho it were Gold in the Hand from which he receiv'd it, will be but Leaves and Dust when it comes to use."—Locke's "Essay on the Human Understanding," Bk. I., Ch. IV. § 23.

PREFACE TO THE FOURTH EDITION.

In the present edition the subject matter and arrangement of the previous editions have been retained. In particular the treatment of the subject of blood coagulation has remained unaltered, unorthodox though it still is from the point of view of the standard text-books. But since the orthodox teaching has been dismissed by a distinguished physiologist in his recent text-book as meaning "little more than inventing names," no apology is needed on my part. For the treatment of the subject given in this course I claim that the essential stages of the process can be demonstrated to the student without postulating hypothetical substances or inventing names. They can be referred to things which can be isolated and seen. Further, by treating the blood platelets as living protoplasm and not as a precipitate of dead colloid material, and by making the changes of these living elements the centre of the problem, all the known facts arrange themselves into an orderly and easily comprehensible sequence of events which follow the two simple "laws of blood coagulation" given on page 37. And, lastly, it links physiological teaching to the problems of medicine. There are in medicine few pathological lesions which exceed in frequency or importance those of thrombosis and its sequelæ. The student who has been taught to look upon platelets as living and particularly fragile protoplasm which exhibits characteristic responses to slight changes in the surrounding medium, will have no difficulty in establishing the connection between the *disintegration* of platelets, which results from a gross injury to the vessel wall, setting in train the extensive process of clotting, and the *agglutination* of platelets following upon a slight injury to the vessel wall, which leads to the local formation of a white thrombus. Again, there is an obvious connection between the great diminution of the platelets in certain blood diseases, such as purpura hæmorrhagica, its

association with delayed clotting of the blood, the experimental production of purpura by the injection of an anti-platelet serum and the importance which we attribute to the function of the platelets in normal blood.

A new test for glucose is given which is capable of demonstrating the traces of glucose normally present in urine. Recent work indicates that slight variations in the amount normally excreted may prove to be of clinical importance. For that purpose this new test, described by me elsewhere some years ago, may be found to be of value.

Two extensive additions have been made. I have always felt it to be a serious defect that the respiration of the living cell, the most fundamental chemical process exhibited by it, could not be readily demonstrated in an elementary course. I believe that the simple experimental arrangement, which was devised by Dr A. H. Drew, will remedy this defect and furnish the student with a number of instructive and striking illustrations.

A section has been added on the balance between acids and bases in the organism. The theoretical conceptions involved in the treatment of this subject are somewhat complex for an elementary course for medical students, whose training in theoretical chemistry is necessarily limited. But the facts from which these conceptions have been evolved are simple and can be readily verified and demonstrated even in an elementary course by the simple experiments given in this section. It is hoped that these experiments will help the student to grasp the essential facts and encourage him to read the subject in his text-books without being frightened by the scarecrows of negative logarithms, dissociation constants, and other learned terms.

The table "Range of Indicators," is a simplified modification of similar tables constructed by Walpole and by Cole.

London, *May* 1920.

PREFACE TO THE FIRST EDITION.

In writing this book for the use of medical students the author has departed from the method usually followed. The subject matter is practically limited to experiments and deductions from the experiments, and is not meant to supply the full and ordered information obtainable from a text-book of physiological chemistry. The arrangement of the work differs from that generally followed, in that the student is at the outset provided with substances familiar to him, such as a potato, an egg, lard, butter, etc. From these he prepares, by simple chemical manipulations, the proximate constituents and their decomposition products, and studies their chemical reactions and physical properties. In this way he is introduced to the subject without interposing complex chemical conceptions, which the usual arrangement of dividing the subject into the study of carbohydrates, fats, and proteins necessarily involves.

In teaching practical physiological chemistry to students who have had only an elementary training in organic chemistry, it seems on the whole more satisfactory and more scientific to impose no tax on the faith of the pupil, even if the work covered is less comprehensive than it might otherwise be. The knowledge acquired in such a course as outlined in this book is at least real, and questions beyond the scope of personal demonstrations by the student may well be left to treatment in lectures.

In order to induce the student to record his own

observations, to draw his conclusions from them, and to correlate the facts observed in the laboratory with the theoretical matter taught in the systematic lectures or in the text-books, the text is interspersed with questions which the student is supposed to answer.

In describing the experiments special care has been taken to refer to the numerous technical details that must be observed and the fallacies that must be excluded in order to obtain a trustworthy result.

Physiology Department,
University of Edinburgh,
March 1912.

CONTENTS.

PART I.

ANIMAL AND VEGETABLE TISSUES AND FLUIDS.

PAGE

POTATO - - - - - - - - - - 1
Preparation of Starch from Potato

COMPLETE HYDROLYSIS OF STARCH BY ACIDS. FERMENTATION OF GLUCOSE - - - - - - 2
Preparation of Glucose and Alcohol from Starch

PARTIAL HYDROLYSIS OF STARCH BY DILUTE ACIDS AND BY FERMENTS - - - - - - - 9
Preparation of Dextrin and Maltose from Starch

LARD, OLIVE OIL - - - - - - - 10
Solubility of Fats

HYDROLYSIS OF FATS BY ALKALI. SAPONIFICATION - 10
Fatty Acids of Lard. Emulsification of Fats

EGG - - - - - - - - - - 12
Osmosis. Lipoids of Egg Yolk. Proteins of Egg-White
General Protein Reactions

MILK - - - - - - - - - - 19
Separation of Constituents. Preparation of Caseinogen and Lactose
Milk Fat. Its Fatty Acids. Comparison of Lactose with Glucose, Maltose, and Cane Sugar. Clotting of Milk by Rennet

FLOUR AND BREAD - - - - - - - 23
Proteins and Carbohydrates of Flour and Bread

MUSCLE AND GLAND - - - - - - - 24
Proteins of Muscle and Gland

LIVER - - - - - - - - - - 25
Carbohydrates of Liver. Glycogen. Iron in Liver

NERVOUS TISSUE - - - - - - - 27
Separation of Lipoids of Nervous Tissue : Cholesterin, Phosphatides, Cerebrosides and Phospho-Cerebrosides
Histochemistry of Lipoids

BLOOD - - - - - - - - - - 31
Coagulation of Blood. Laws of Blood Coagulation
Hæmolysis. Oxidising Ferment of Blood. Oxygen-Capacity of Blood
Chemistry of Blood Pigment. Absorption-Spectra of Hæmoglobin and its Derivatives
Chemistry of Blood-Serum. Separation of Globulin and Albumin. Constituents other than Proteins

METHOD OF CHEMICAL EXAMINATION OF TISSUES - - 50

PART II.

DIGESTION.

PAGE

SALIVA - - - - - - - - - 53

GASTRIC DIGESTION - - - - - - 55
Gastric Juice
Gastric Contents—1. Acids. 2. Products of Peptic Digestion

PANCREATIC DIGESTION - - - - - 62
Pancreatic Juice
Products of Tryptic Digestion

METHOD OF TESTING FOR FERMENTS—CONTROL EXPERIMENTS - - - - - - - 63

BILE - - - - - - - - - 64
Effect of Bile on Emulsification of Fats. Bile Salts. Bile Pigments

PART III.

NORMAL METABOLISM.

TISSUE RESPIRATION - - - - - 68
Oxygen Absorption by Tissue Cells. Warm and Cold Blooded Animals. Action of Narcotics. Antagonism between Na-and Ca Ions. Carbondioxide Excretion

URINE - - - - - - - - - 71
Constituents of Normal Urine
Quantitative Estimation of certain Nitrogenous Constituents: Total Nitrogen, Urea, Ammonia
Significance of Nitrogen Distribution in Urine
Quantitative Estimation of Chlorides

PATHOLOGICAL METABOLISM.

GASTRIC CONTENTS - - - - - - 96
Quantitative Estimation of Hydrochloric Acid. "Free," "Physiologically Active," and "Combined" Hydrochloric Acid

ABNORMAL CONSTITUENTS OF URINE - - - - 99
Proteins
Reducing Sugars. On the Presence of Glucose in Normal and in Abnormal Urines. Estimation of Glucose
Aceto-Acetic Acid and Acetone. Method of Examining the Urine in Cases of Acidosis
Blood Pigment
Bile
Pathological Urinary Deposits

FORM FOR REPORT ON URINE ANALYSIS - - - 122

BALANCE BETWEEN ACIDS AND BASES IN THE ORGANISM - 124
Definition of Acidity and Alkalinity. Notation of Hydrogen-Ion Concentration. Indication of Hydrogen-Ion Concentration by Indicators. Range of Indicators, "The Buffer Effect." Estimation of Hydrogen-Ion Concentration of Urine and other Body Fluids

TABLE OF RANGE OF INDICATORS - - - - 134

INDEX - - - - - - - - - 135

Directions for a Practical Course in Chemical Physiology.

ANIMAL AND VEGETABLE TISSUES AND FLUIDS.

POTATO.

Experiment 1. Preparation of Starch from Potato.—Scrape a potato as finely as possible with a knife, collect the scrapings in a beaker, stir with about 100 c.c. water, and strain the water which contains a large part of the starch through a piece of unbleached muslin. Mix the residue with more water and strain again. After repeating this with several portions, collect the water in one vessel and allow the starch grains to settle to the bottom. Examine some starch grains microscopically; observe the effect of adding a drop of very dilute iodine solution, and sketch. Pour off the water, add more, and allow again to settle, repeating till the starch appears white.

Experiment 2. Preparation of Starch Solution.—Rub 1 gm. of starch to a thin paste with a little water, and pour the mixture slowly, with constant stirring, into 150 c.c. boiling water. Use the solution so obtained for Experiments 3-18.

Tests for Starch.

Experiment 3.—(*a*) To some of the starch solution in a test tube add a drop of dilute iodine solution. Note the result. (*b*) Heat the tube gradually. What change takes place? (*c*) Allow the solution to cool, and observe the result. (*d*) Make alkaline with a few drops of sodium hydroxide. Note the result. (*e*) Neutralise with a few drops of dilute hydrochloric acid. Explain the result.

Experiment 4.—Repeat the preceding test, first adding sodium hydroxide solution before testing with iodine. Record and explain the result.

Experiment 5.—To 5 c.c. (roughly a quarter of a test tube) of the starch solution add three times its volume of saturated ammonium sulphate solution. Shake, and allow to stand for fifteen to thirty minutes. Filter and test separately the precipitate and the clear filtrate with iodine for starch.

Experiment 6.—To 5 c.c. of the starch solution add alcohol until a precipitate appears. Apply the iodine test for starch to the precipitate, and to the filtrate separately, and note the result.

COMPLETE HYDROLYSIS OF STARCH BY ACIDS. FERMENTATION OF GLUCOSE.

PREPARATION OF GLUCOSE AND ALCOHOL FROM STARCH.

Preparation of Glucose by Acid Hydrolysis of Starch.

Experiment 7.—To 100 c.c. of starch solution, in a flask, add about 3 c.c. of concentrated hydrochloric acid, and boil continuously over a small flame. Add occasionally

some water, previously heated in a test tube, to replace the water which has evaporated. (*a*) What change do you observe in the appearance of the starch solution? Remove every five minutes about 5 c.c. of the solution by means of a pipette, place in a test tube, cool, and test with a drop of dilute iodine solution. (*b*) Record the results obtained in each case. Continue boiling the bulk of the solution in the flask until the iodine test is negative, *i.e.*, until a drop of dilute iodine solution, when added to about 5 c.c. of the solution, removed from the flask and cooled, fails to produce a colour. This point will be reached after about thirty minutes; the time of boiling required varies with the concentration of the starch paste and of the acid. The solution thus obtained is used for Experiments 8-16. (*c*) What has taken place?

Preparation of Alcohol by Fermentation of Glucose.

Experiment 8.—(*a*) To about 15 c.c. of the glucose solution obtained in Experiment 7 add dilute sodium hydroxide until the reaction is almost neutral to litmus, but still slightly acid. Add a small quantity of fresh yeast, and gently shake so as to break up the yeast. With the emulsion of yeast in the glucose solution fill the closed limb of a fermentation tube, so that no air bubble is left, and place the tube for about an hour in a warm water-bath at 40° C. What takes place?

(*b*) Put the fermentation tube into the cupboard. After twenty-four hours remove the solution and filter. Examine the filtrate for alcohol by means of the iodoform test and the bichromate test. [*Tests for alcohol*—(*a*) *Iodoform test.* To the solution add a solution of iodine in potassium iodide until it is brown, then add just sufficient sodium hydroxide to decolorise the mixture. Warm gently. A yellow crystalline precipitate with the characteristic smell of

iodoform is formed. (*b*) *Bichromate test.* Dissolve a crystal of potassium bichromate in the solution and add a little dilute sulphuric acid. Heat. The solution turns green. and the characteristic odour of aldehyde will be observed, What reaction has taken place ?]

Carefully neutralise, with sodium hydroxide, the rest of the solution obtained in Experiment 7, and perform the following tests for glucose.

Experiment 9.—(*a*) To 5 c.c. of sodium hydrate in a test tube add a solution of copper sulphate. A blue flaky precipitate forms which does not dissolve on shaking. What is it ? Boil. Describe the result.

Explain and compare with the following :—

Tests for Glucose.

(*b*) **Trommer's Test.**—Add to 5 c.c. of the glucose or sugar solution an equal volume of strong sodium hydrate or potassium hydrate solution, then a dilute solution of copper sulphate, drop by drop, shaking after each addition, as long as the bluish precipitate formed continues to dissolve readily. If an excess of $CuSO_4$ is added, the precipitate remains, and must be dissolved by adding a few crystals of sodium potassium tartrate (Rochelle salt). The result is a deep blue solution. Why is no precipitate formed in this case ? Heat the solution, and note the result. Explain the chemical changes which have taken place.

Experiment 10. Fehling's Test.—Prepare Fehling's solution. Dissolve in 80 c.c. of distilled water 7 gm. of copper sulphate, and label solution "A." Dissolve 34·5 gm. sodium potassium tartrate in 120 c.c. sodium hydroxide 10 per cent., and label solution "B." Mix "A" and "B," and fill the mixture into bottle labelled "Fehling's Solution."

Boil 5 c.c. of Fehling's solution in a test tube (it should remain clear), and then add a few drops of the glucose solution. Let it stand a while and note the result. Repeat the test without heating.

Experiment 11. Caramelisation (Moore's Test).—Boil 5 c.c. of the glucose solution with a little caustic soda. The solution turns yellow owing to the formation of caramel. Boiling with alkaline salts, *e.g.*, sodium carbonate, also induces caramelisation.

Experiment 12. Böttger's Test.—To a quarter of a test tube of the glucose solution add a little solid bismuth subnitrate, and about double the quantity of sodium carbonate. Heat and keep boiling for two minutes. Describe and explain the result.

Experiment 13. Nylander's Test.—Prepare Nylander's reagent. Dissolve 4 gm. sodium potassium tartrate and 2 gm. bismuth subnitrate in 100 c.c. sodium hydroxide 10 per cent. Pour the reagent into bottle labelled "Nylander's Reagent." Add one part of Nylander's reagent to ten parts of sugar solution, and keep boiling over a small flame for two minutes. Compare with Böttger's test.

Repeat tests 9 to 13 with a very dilute glucose solution in order to test their delicacy. Tests 9 to 13 are reduction tests. Why are they called so, and what is the rationale of these tests? What happens if these tests are applied to starch solution?

Experiment 14. Osazone Test.—To about 10 c.c. of the glucose solution add 10 drops of phenylhydrazine and an equal amount of glacial acetic acid. Shake the test tube and keep in the boiling water-bath for $\frac{1}{2}$ to 1 hour. Allow to cool slowly and examine crystals under the microscope, and sketch. They consist of glucosazone.

Experiment 15. Molisch's Test.—To a few c.c. of the solution add a few drops of an alcoholic solution of α-naphthol. Incline the test tube and allow about 5 c.c. of concentrated sulphuric acid to flow down the side of the tube so that the acid settles at the bottom of the tube. At the junction of the two liquids a green ring may at first be formed (if traces of nitrates are present in the sulphuric acid), and above this, in a short time, a reddish-violet ring. The green ring, if present, must be disregarded. If the two liquids be mixed and cooled by gently shaking in running water, so that excessive heating is prevented, the mixture assumes a bluish-red or dark blue colour. [All carbohydrates and some proteins give this reaction. The test is used to demonstrate the presence of a carbohydrate group in proteins.]

Use of Polarimeter.

Experiment 16. Optical Activity. Polarimeter.—Examine by means of a polarimeter the effect of the glucose solution on polarised light. Proceed as follows.[1]

Examine the polarimeter. It consists of a hollow brass tube which, at the one end, contains a Nicol prism, the "polariser," together with a double quartz plate. At the other (upper) end of the brass tube and attached to it by means of a hinge and a spring catch, is another Nicol prism, the "analyser," together with a small telescope and a graduated arc divided into whole degrees. The "analyser" can be rotated by means of a milled head. The rotation can be read accurately to a tenth of a degree by means of a vernier.

Lock the hinged part and place the instrument so that

[1] The following description applies to a polarimeter of the Soleil type, and more particularly to the hand-polarimeter supplied by Winkel, Göttingen.

light falls into the "polariser" from a white surface. Make the following preliminary observations :—

1. Rotate the analyser so that the vernier reads zero. On looking through the instrument the field of vision will appear as a circle divided by a straight line into two halves, which have *exactly* the same violet tint. Move the small telescope so that the dividing line is sharply focussed.

2. By means of the milled head, rotate the analyser slightly to the right and to the left. Note that a very slight rotation of the analyser is sufficient to produce a marked contrast in colour between the two halves of the field, the one half becoming red, the other blue.

3. Rotate the analyser by 90° to the right. Note that both fields are again tinted alike, but the tint is now yellow. Rotate slightly to the right and to the left. Note that now slight rotations of the analyser do not produce a marked contrast in colour between the two halves of the field, *i.e.*, the yellow tint is not so sensitive in indicating rotation as the violet tint.

4. Starting again from zero, rotate the analyser through 360°. Note that the two halves of the field are always unequally tinted except at four points of the circle : at 0° and at 180° the field shows in both halves the same sensitive violet tint ; at 90° and at 270° the field shows in both halves the same non-sensitive yellow tint.

Note that in order to take a reading the instrument must be set so that—

1. *The dividing line between the two halves of the fields is sharply focussed by means of the telescope.*

2 *The two halves of the field show exactly the same sensitive violet tint, the so-called "critical colour."*

After these preliminary observations insert a tube filled with a solution of glucose (about 5 per cent.). The observation tube must be clean and completely filled with the solution, so that air bubbles are excluded. Release the spring catch, which holds the part containing the analyser in position, insert the observation tube, and lock the instrument. The light, after having passed through the "polariser," and having become polarised, must now traverse the liquid in the observation tube before it reaches the analyser. Place the vernier on zero, and focus the telescope. Note that now the two halves of the field show a marked contrast, one half being blue, the other red. Rotate the analyser slightly until the two halves of the field show exactly the same violet tint (the critical colour). Note the position of the vernier; it will be to the right of the zero point. The solution of glucose is, therefore, "optically active," and has rotated polarised light to the right: it is "dextro-rotatory."

Compare the effect produced by a solution of glucose with that produced by other organic substances, urea for instance. Insert a tube containing a 5 per cent. solution of urea, and place the vernier on zero. Note that the two halves of the field remain equally tinted. The solution of urea is "optically inactive."

PARTIAL HYDROLYSIS OF STARCH BY ACIDS.

PREPARATION OF DEXTRIN.

Preparation of Dextrin by Acid Hydrolysis of Starch.

Experiment 17.—Proceed as in Experiment 7, but add only ten drops of concentrated hydrochloric acid to 100 c.c. of starch paste. Remove every three minutes about 5 c.c.

of the solution, cool, and test with dilute iodine solution. Discontinue the boiling when a purple or red colour is obtained with iodine. The solution then contains erythrodextrin. To the remainder of the solution apply Trommer's, Fehling's, and Nylander's tests. Record your results.

PARTIAL HYDROLYSIS OF STARCH BY FERMENTS.

PREPARATION OF DEXTRIN AND MALTOSE.

Preparation of Dextrin and Maltose by Action of Saliva and Starch.

Experiment 18.—Prepare some dilute saliva by rinsing out the mouth for one minute with 5 c.c. warm water. Collect the washings in a beaker. Repeat. Filter the dilute saliva. Place a series of drops of iodine on a porcelain plate. Put some starch solution (about 5 c.c.) in a test tube and add about half a test tubeful of saliva. Shake the mixture and place in the water-bath at 40° C. Every minute take out a drop with a glass rod and apply it to one of the iodine drops on the porcelain slab. What changes take place in the mixture? Keep the mixture in the water-bath for $\frac{1}{2}$ to 1 hour, until all the starch has been transformed into maltose. This solution is used for Experiment 19. By what constituent of the saliva is starch transformed into maltose?

Tests for Maltose.

Experiment 19.—With the solution of maltose obtained in Experiment 18 carry out the tests 9 (*b*) to 14. Note the results. How does maltose differ from glucose, and how can it be distinguished from it?

LARD, OLIVE OIL.

Solubility of Fats.

Melt a little lard in a porcelain basin on a boiling water-bath; with the melted fat carry out the following experiments:—

Experiment 20.—Add a few drops of the lard to each of five test tubes containing (1) acetone, (2) alcohol, (3) ether, (4) chloroform, (5) water, and note the solubility of the fat in these solvents.

Experiment 21.—Let a drop of the alcoholic or ethereal solution fall on a piece of white paper, and note the grease spot which remains after the solvent has evaporated.

Experiment 22.—To a test tube containing water add a few drops of the alcoholic solution of the fat. A precipitate appears. Explain.

Experiment 23.—Repeat Experiments 20 to 22 with olive oil instead of lard.

HYDROLYSIS OF LARD BY ALKALI (SAPONIFICATION).

PREPARATION OF FATTY ACIDS.

Preparation of Fatty Acids by Hydrolysis of Fats.

Experiment 24.—Slowly add about 5 c.c. of the melted lard to 50 c.c. of an alcoholic solution of caustic potash, contained in a flask, and kept on a boiling water-bath. Mix thoroughly and heat for ten to twenty minutes. Add a few drops of the mixture to some water in a test tube as in Experiment 22. No oil globules separate out. Why? (If oil globules are still seen, the reaction is not complete) and heating must be continued with more alcoholic soda., When the reaction is complete, slowly pour the solution

in the flask into a beaker containing 100 c.c. of warm water and mix thoroughly. To the watery solution (which contains soap) add some 25 per cent. sulphuric acid (one part concentrated sulphuric acid slowly added to three parts of water), and heat on the water-bath until the melted fatty acids separate out as an oily layer floating on the top of the liquid. Cool. The fatty acids solidify, and can then be removed and freed from adhering sulphuric acid by rinsing with cold water.

Properties of Fatty Acids and of Soaps.

Experiment 25.—Suspend some of the fatty acids in water and add dilute sodium hydroxide. The fatty acids dissolve. Soap is formed again. What is soap? Use the solution of soap thus obtained to demonstrate the following properties:—

(*a*) Shake up some of the soap solution with warm water. A soap lather is produced.

(*b*) To some soap solution add solid sodium chloride until the solution is saturated. The soap is precipitated: "salted out."

(*c*) Add a few drops of calcium chloride solution to some of the soap solution. A precipitate is formed. Of what?

The remainder of the soap solution is used to demonstrate the part played by soap in—

EMULSIFICATION OF FATS.

Experiment 26.—Label two test tubes "(*a*)" and "(*b*)." Place in (*a*) some water and in (*b*) some soap solution. Add to each three drops of olive oil and shake. A permanent emulsion is formed in (*b*) but not in (*a*). Explain.

Experiment 27.—The same result is, of course, obtained if neutral fat containing some fatty acid is shaken up with a little dilute alkali solution. For instance, to half a test tubeful of water add one drop of a 10 per cent. solution of sodium hydroxide and 2 c.c. of ordinary olive oil, which always contains some free fatty acid. An emulsion is formed. Explain.

A neutral fat, which does not contain any free fatty acids, will not give a permanent emulsion with dilute sodium hydroxide. This reaction may, therefore, be used to test for the presence of free fatty acids in a fat. These are present if a fat is rancid.

Another method of detecting whether a fat is rancid is as follows :—

Experiment 28.—To some alcohol in a test tube add a drop of phenolphthalein and one or two drops of very dilute caustic soda, just enough to produce a red colour. Add this fluid to a solution of olive oil in alcohol. If fatty acids are present the red colour disappears. Why?

EGG.

Demonstration of Osmosis.—Three eggs, from which the shells have been removed by immersion in hydrochloric acid, are weighed. The one is immersed in distilled water, the other in a 0·9 per cent. salt solution, the third is immersed in glycerine. Note the change in volume and weight.

Demonstration of a semi-permeable membrane.

Separation of Constituents of Egg.—Break a raw egg and collect separately in two beakers the white and the yolk of the egg. The *white of the egg* consists mainly of *proteins*, while the *yolk*, besides other protein substances and some fat, contains *lipoids* (*e.g.*, lecithin, cholesterin).

Lipoids of Egg-Yolk.

Experiment 29. Lipoids of Yolk. Preparation of Lecithin and Cholesterin.—Mix the yolk thoroughly with about 20 c.c. of ether, pour into a flask, and close flask. Shake vigorously, and allow it to stand for some time. (In the meantime the solution of egg proteins may be prepared as directed below in Experiment 32.)

(N.B.—*In working with ether all gas flames in the neighbourhood must be extinguished.*)

Pour off from the residue the ethereal, deeply coloured solution into a porcelain capsule, and extract the residue again with 20 c.c. of ether. The ethereal extracts are concentrated to a small volume by placing the capsule in the fume chamber in a previously heated water-bath (use no flame!). Acetone is then added in excess until a distinct precipitate is formed. The precipitate is a crude mixture of phosphorised fats. This mixture is frequently described by the name of its main constituent—"lecithin." Remove the precipitate by filtration. The filtrate, which contains cholesterin, is evaporated on the water-bath. Cholesterin separates out, admixed with some lecithin.

(*a*) Lecithin.

Experiment 30.—Dissolve in alcohol the precipitate on the filter. The bulk of it dissolves. Drop the alcoholic solution into water and stir. A white precipitate of "lecithin" is formed. Proceed in the same way, but drop the alcoholic solutions of lecithin into ether. Compare the solubility of lecithin in water, acetone, alcohol, and ether with that of ordinary fats in the same solvents, and record.

Boil the watery emulsion with caustic soda. It becomes clear. Notice the smell of trimethylamine. On acidifying, fatty acids separate out. Explain the change which has taken place.

For further tests for lecithin (demonstration of phosphoric acid in its molecule), see Experiment 61.

(*b*) Cholesterin.

Experiment 31.—Remove all the water from a test tube by washing it out with alcohol and ether. Place some chloroform in this dry test tube, and dissolve in it some cholesterin. Add an equal bulk of concentrated sulphuric acid, and shake gently. The chloroform solution which rises to the top turns red, the acid at the bottom of the test tube shows a green fluorescence (Salkowski's test).

The necessity for using a dry test tube is shown by the fact that the red colour of the chloroform solution disappears if it is poured into a wet test tube.

For further tests for cholesterin (crystalline form), see Experiment 60.

Proteins of Egg-White.

Experiment 32. White of Egg.—Cut up the egg-white with a pair of scissors. The viscid fluid thus obtained is faintly yellow in colour, alkaline in reaction, and has a specific gravity of 1·045. It contains about 10 per cent. of proteins; the greater proportion is egg albumin.

A portion of the white of egg (about 5 c.c.) is diluted with nineteen times its volume of water. A slight, but well-marked, precipitate of a protein appears, but the bulk of the protein remains in solution. The precipitate readily dissolves if the concentration of salts, which by dilution with water has been greatly lowered, is slightly raised again by the addition of a few drops of a saturated solution of sodium chloride. In order to distinguish the proteins which remain in solution when the concentration of salts is lowered from those, otherwise similar, proteins which fall out under these conditions, the former are called

albumins, the latter *globulins*. A more detailed study of their behaviour and solubilities will be made later, when the composition of blood-serum is being examined, because in serum they are present in about equal proportions, while in the white of egg the amount of globulin present is relatively small.

The globulins and albumins are the most typical and widely distributed groups of "simple proteins." They show the property of being coagulated by heat.

Heat Coagulation.

Experiment 33. Heat Coagulation of Albumins anp Globulins.—

(*a*) Heat 5 c.c. of the diluted egg-white in a test tube to boiling point. The solution becomes opalescent, but there is no definite coagulum.

(*b*) Heat 5 c.c. with 1 to 2 drops of dilute acetic acid. A coagulum is formed.

(*c*) Heat 5 c.c. with 2 drops of glacial acetic acid. No coagulum occurs. Acid albumin is formed, which does not coagulate on boiling.

(*d*) Heat 5 c.c. with 2 to 3 drops of dilute sodium carbonate solution. There is no coagulation. Alkali albumin is formed, which does not coagulate on boiling.

The globulin and albumin of egg-white is thus only coagulated by boiling when the solution is neutral or faintly acid. If the solution be alkaline as in (*d*), the proteins are acted on by the alkali, as the temperature rises, and converted into *alkali albumin*. If strongly acid as in (*c*), the proteins are converted into *acid albumin*.

These compounds, which are grouped together as *meta-proteins*, are not coagulated on heating their solutions.

They are precipitated on neutralising their solutions, and dissolve again in an excess of either acid or alkali.

With the diluted egg-white, which represents approximately a 0·5 per cent. solution of ovalbumin and ovoglobulin, carry out the following tests, by which proteins can be recognised.

GENERAL PROTEIN REACTIONS.

General Protein Tests.

Experiment 34. Colour Tests :—

(*a*) *Biuret Test.*—To a portion of the protein solution add sodium hydroxide so that the solution is strongly alkaline, then one or two drops of very dilute copper sulphate solution. (An excess of copper sulphate must be avoided.) The solution becomes violet.

On what group in the protein molecule does this test depend? Prepare some biuret by heating a few urea crystals in a dry test tube until the melted mass begins to solidify again. Allow to cool, dissolve in a little water, and apply the biuret test. What is the structural formula of biuret?

(*b*) *Millon's Test.*—To a portion of the protein solution add a few drops of Millon's reagent (solution of mercurous and mercuric nitrates). A precipitate forms, which, on heating, becomes brick-red. The red colour constitutes the essential part of the test.

On what group in the protein molecule does this test depend? Repeat with a dilute solution of phenol.

Apply Millon's test to the protein solution after having added some sodium chloride. What takes place? Explain.

Millon's test can be applied to insoluble proteins.

(*c*) *Xanthoproteic Test.*—To a few c.c. of the protein solution add one-third of its volume of strong, pure, nitric

acid; a white precipitate may or may not be produced (according to the concentration and nature of the protein). Boil. The precipitate or liquid turns yellow. Cool the test tube, and carefully add excess of ammonia, so as to form a layer above the nitric acid. An orange colour is produced at the junction. This constitutes the essential part of the test. Mix the ammonia with the acid by shaking, and note that the yellow colour of the solution deepens.

On what group in the protein molecule does this test depend? Repeat the test with benzene.

This test can be applied to insoluble proteins.

Experiment 35. Precipitation by Strong Mineral Acids (Nitric Acid or Heller's Test).—Place 5 c.c. of the protein solution in a test tube, and by means of a pipette add 1 or 2 c.c. of strong pure nitric acid very carefully to the bottom of the solution, so that it forms an under layer. A white ring of coagulated protein appears at the junction of the two fluids. (Allow ten minutes if the reaction is slow in appearing.)

Experiment 36. Precipitation by Alkaloidal Reagents.—Proteins in acid solution are precipitated by reagents which precipitate alkaloids; *e.g.*:—

(*a*) *Hydroferrocyanic Acid.*—Make a portion of the protein solution distinctly acid with acetic acid; then add a few drops of potassium ferrocyanide solution. A precipitate is formed. Why is it necessary to carry out this test in this way, instead of adding free hydroferrocyanic acid to the protein solution?

(*b*) *Picric Acid.*—To some protein solution add picric acid drop by drop. Note that a precipitate forms around each drop as it falls in. On shaking, this precipitate

dissolves at first. If sufficient picric acid has been added the precipitate remains.

(*c*) *Salicylsulphuric Acid.*—To a portion of the protein solution add a few drops of salicylsulphuric acid solution. A precipitate is formed.

A number of other alkaloidal reagents (tannic acid, phosphotungstic acid, etc.) give similar precipitates with protein solutions.

What is an alkaloid? Why do albumins and globulins in acid solution behave like alkaloids towards these reagents?

Experiment 37.—Test the delicacy of the protein tests 33 to 36 by applying them to (*a*) 2 c.c. egg-white diluted with 98 c.c. water (1 : 50 dilution), (*b*) 1 c.c. egg-white diluted with 99 c.c. water (1 : 100 dilution). Record your results.

Experiment 38. Precipitation by Alcohol.—To the protein solution add an excess of alcohol. A precipitate is formed. Allow the precipitate to stand in contact with alcohol for half an hour. Decant the alcohol and add water to the precipitate. It has become insoluble in water, having been coagulated by the alcohol. What is the difference between "precipitation" and "coagulation"?

Testing for Carbohydrates in Presence of Proteins.

Experiment 39. Carbohydrate Material of White of Egg.—Egg-white does not contain any polysaccharide of a starch-like nature or a reducing sugar in the free state.

Demonstrate this by applying the iodine test, Fehling's and Nylander's tests to the diluted egg-white solution, from which all proteins have previously been removed by heat coagulation. Record. *Note that the tests for carbohydrates can only be applied after all proteins have been removed.*

Test with the biuret test for the absence of proteins before applying the tests for carbohydrates.

If, however, Molisch's test (Exp. 15) is applied to the egg-white solution (still containing proteins), a positive result is obtained. This shows that while no free carbohydrate is present in the white of egg, the protein of egg-white contains a carbohydrate group chemically bound in its molecule.

MILK.

Experiment 40.—Test the fresh milk with litmus paper. The reaction is either faintly alkaline or amphoteric (*i.e.*, turns blue litmus red and red litmus blue).

Determine the specific gravity of milk. Record.

Why has skimmed or separated milk a higher specific gravity than fresh milk?

Fat.

Experiment 41. Separation of Constituents.—Extract the fat by shaking 5 c.c. of milk in a test tube with twice its volume of ether. Pour off the ether and allow to evaporate until only a few drops are left. Pour these on to a filter paper. A greasy spot indicates the presence of fat.

Caseinogen.

Dilute 20 c.c. of milk with 80 c.c. of water. Add carefully dilute acetic acid drop by drop, stirring the fluid after each drop has been added, until a flocculent precipitate is formed, and the solution in which it floats appears clear. An excess of acid must be carefully avoided, as it would dissolve the precipitate. The precipitate consists of caseinogen—the main protein of milk—with adherent fat. Filter. With the precipitate, carry out the experiments described below under caseinogen (Exp. 43).

Lactalbumin, Lactoglobulin.

The filtrate from the caseinogen, which still gives the tests for proteins, contains (besides small amounts of lactalbumin and lactoglobulin) lactose—the carbohydrate material of milk. Remove the albumin and globulin by heat coagulation in the following way :—

Carefully neutralise the acid filtrate with dilute sodium carbonate solution. Heat the neutral solution to boiling point, and add a few drops of dilute acetic acid. If the heat coagulation is carried out correctly, the lactalbumin and lactoglobulin separate out as a flocculent precipitate which is removed by filtration, and the filtrate does not give any tests for protein.

Lactose.

When all the protein has been removed, the filtrate may be used for the experiments described below under lactose. It contains, besides lactose, inorganic phosphates of calcium, which can be detected by the ordinary test for phosphates. Label the filtrate "*Lactose.*"

For the examination of milk fat butter is given out.

Experiment 42. Milk Fat, its Fatty Acids.—Different fats differ in the nature of the fatty acids which are combined with glycerine. Liberate the fatty acids of milk fat by saponification (see Exp. 24). Proceed as follows :—

Heat a little butter with a small quantity of alcoholic soda until a clear yellow solution is obtained. Pour the solution into a beaker containing hot water (no oil drops should be seen) and heat to expel the alcohol. Then acidify with dilute sulphuric acid, warm again, and note the smell of butyric acid and caproic acid. Compare briefly butyric acid and the higher fatty acids obtained from lard with regard to their solubility in water, their volatile nature, and their molecular weight.

Experiment 43. Caseinogen.—Dissolve the precipitate of caseinogen obtained in Experiment 41 in dilute sodium carbonate solution. The adherent fat remains suspended. Filter through a wet filter paper, and apply to the caseinogen solution the colour tests for proteins. Record.

Determine whether caseinogen is a protein coagulable by heat (see Exp. 33). Record.

Experiment 44. Lactose.—Evaporate some of the filtrate obtained in Experiment 41, and labelled "Lactose," to a syrup on the water-bath, and allow to stand in the cold until the sugar has crystallised out.

To another portion of the filtrate labelled "Lactose" apply the following tests for sugar:—

Fermentation test (No. 8).

Reduction tests: Trommer or Fehling (Nos. 9 and 10); Böttger or Nylander (Nos. 12 and 13).

Osazone test (No. 14).

Cane Sugar.

Experiment 45. Comparison of Lactose with Cane Sugar.—Apply reduction tests, fermentation test, and osazone test to a solution of cane sugar which is given out. Record your results.

Boil 5 c.c. of cane sugar solution with five drops of concentrated hydrochloric acid for two minutes. Neutralise with caustic soda, and apply Fehling's and Nylander's tests. Record your results.

Experiment 46. Action of Different Disaccharides on Polarised Light before and after Hydrolysis.—Examine solutions of lactose, maltose, and cane sugar of equal concentration in a polarimeter. Proceed as described in Experiment 16.

Hydrolyse solutions of lactose, maltose, and cane sugar

by boiling for five minutes with five drops of concentrated hydrochloric acid, and examine solutions of hydrolysed sugars again. Record your results. Why is hydrolysed cane sugar called "invert-sugar"?

From Experiments 8, 9, 10, 12, 13, 14, 16, 19, 44, 45, and 46, construct the following table:—

Reactions of Sugars.

	Nature of Sugar.	Trommer or Fehling.	Nylander.	Optical Activity.	Fermentation.	Form of Osazone.
Monosaccharide	Glucose -					
Disaccharide	Maltose -					
	Lactose -					
	Sucrose - (Cane Sugar)					

Verify the table by applying the various tests to the solutions of the different sugars which are given out.

Action of Rennet on Milk.

Experiment 47. Clotting of Milk.—To 5 c.c. of milk in a test tube add 1 c.c. of a neutral solution of rennet ferment (prepared by extracting a calf's stomach with glycerine). Place the mixture in a water-bath, kept at 37° to 40°. After a few minutes a firm clot forms, from which, on standing, a clear fluid exudes. The clot contains casein and fat. What is casein? The clear fluid—the whey—contains all the other constituents of the milk. Demonstrate their presence by suitable tests and record your results.

Experiment 48. Prove that the Formation of the Clot Depends upon the Presence of Soluble Calcium Salts.—Precipitate all the calcium present in 5 c.c. of milk by adding 2 c.c. potassium oxalate solution (1 per cent.). Then add 1 c.c. of rennet and place in water-bath. No clot forms if all the calcium has been precipitated. Then add to the unclotted milk 2 c.c. $CaCl_2$ solution (2 per cent.). A flocculent precipitate is produced. Explain the results.

FLOUR AND BREAD.

Experiment 49. Flour.—Knead some flour with a little warm water to form a stiff dough, and allow to stand for about fifteen minutes. Place the dough in a muslin bag and continue kneading in a basin of water; starch grains pass through. If the water is poured from the basin into a beaker the deposit of starch grains which settle can be easily seen. Prove their chemical nature by examining them microscopically, and by boiling some grains with water and applying the iodine test to the watery solution thus obtained. Remove the grains from the water by filtration, and test the filtrate with iodine for the presence of dextrin (see Exp. 17), and with Fehling's and Nylander's tests for glucose (Tests Nos. 10 and 13). Continue kneading the dough under the tap until all the starch has been removed. A yellowish sticky mass remains. This is gluten, the chief protein of wheat. Prove its protein nature by applying the ordinary colour tests for solid proteins (No. 34, *b*, *c*).

Experiment 50. Bread.—Grate some bread finely, and extract, first with cold water, then with boiling water. Strain the watery extract through muslin.

Examine :—

1. Cold water solution for glucose, starch grains, dextrin.

2. Hot water solution for glucose, starch, dextrin. In order to demonstrate the presence of dextrin when starch is also present in solution, remove the latter by half saturating the solution with ammonium sulphate. The dextrin passes into the filtrate.

3. The residue is gluten, and can be identified as a protein by the colour tests (No. 34), as in Experiment 49.

Record the results. What difference is there between the carbohydrates of flour and those of bread?

What changes do the carbohydrates of flour undergo in the process of baking, and how do you account for these changes?

Experiment 51.—Extract some crust of bread with water, and test for starch, dextrin, and glucose.

Note that in the crust glucose is present in traces only. Why?

MUSCLE AND GLAND.

Experiment 52. Proteins of Muscle.—In a mortar rub thoroughly 10 gm. of muscle (best from white fish) with 5 gm. of sodium chloride, then to the pulp add 50 c.c. of water, so as to make a 10 per cent. solution of NaCl. Stir the mixture well and strain through muslin. Label the solution, "*Salt Extract* (A)."

Rub the residue with 0·2 per cent. NaOH solution in the mortar and again strain. Label this solution, "*Soda Extract* (B)." A residue containing collagen is left on the muslin.

Salt Extract (A).—Determine that this extract contains protein by applying the colour tests for protein (Exp. 34). Record the results.

Determine that it is a protein coagulable by heat (globulin or albumin), by heating the *neutral extract* with

the addition of a few drops of acetic acid (Exp. 33). (Note the conditions necessary for this test as explained in Exp. 33.) Record the results.

Nucleoprotein.

Soda Extract (B).—On careful addition of acetic acid to this extract a precipitate forms which is redissolved in a large excess of the acid. It is the nucleoprotein of muscle.

Determine that the nucleoprotein solution gives the colour tests for proteins (No. 34).

Experiment 53. Proteins of Glands.—Repeat this experiment with glands (pancreas) instead of muscle. Note and record how these two tissues differ with regard to the relative amounts of (1) heat-coagulable protein (albumin and globulin) (salt extract), (2) nucleoprotein (soda extract).

LIVER.

The student is supplied with—

A. Small pieces of the liver of an animal which has been fed for some time on a diet rich in carbohydrates (carrots). The liver was placed in alcohol immediately after death.

AA. Small pieces of the liver of an animal fed on the same diet, but the liver was placed in alcohol twenty-four hours after death.

AAA. Small pieces of the liver of a fasting animal. The liver was placed in alcohol immediately after death.

B. Pieces of other glands, *e.g.*, kidney, placed in alcohol immediately after death.

With A carry out the following tests:—

Presence of Glycogen in Liver.

Experiment 54. Glycogen.—Cut up a small piece into shreds, place the shreds into a test tube. Add 10 c.c. of water and heat slowly to boiling point. Cool thoroughly. Decant from the shreds and divide the solution into three parts.

1. To one part add two drops of iodine solution. A brown colour results, indicating the presence of glycogen.

Care must be taken not to add too much of the iodine solution, since the iodine solution itself has a similar colour. It is useful, therefore, to carry out a control experiment in a test tube which contains the same amount of water as the glycogen solution to be tested. If two drops of iodine solution are added to the water a yellow colour is obtained.

The brown colour obtained by adding iodine to the glycogen solution disappears on heating, and reappears on cooling. It disappears on making the solution alkaline. It reappears on neutralising the alkaline solution. Compare with starch. (Experiment 3.)

2. To another part apply Fehling's test. No reduction results.

3. A third portion is boiled with dilute HCl for five minutes. Neutralise and apply Fehling's test. Reduction occurs. Explain. To which group of carbohydrates does glycogen belong?

The presence of large amounts of glycogen in a tissue can be demonstrated rapidly by applying iodine directly to the tissue as follows:—

Experiment 55.—To a piece of liver A apply a drop of dilute iodine solution by means of a glass rod. After a minute remove the iodine solution by rinsing the tissue under the tap. A deep brown colour appears where the

iodine has acted. Record your result. Carry out the test with AA, AAA, and B. Record your results. The colour is very faint in AA and AAA, and may even be absent. Why? It is absent in B. Why?

Why must the tissues be preserved in alcohol and not in a watery solution (of formalin, for instance), if one wishes to test for glycogen?

Experiment 56. Test for Iron.—To a test tube containing 10 c.c. of water add a few drops of ferric chloride. To this ferric chloride solution add a few drops of potassium ferrocyanide. A blue colour results. What has been formed?

Explain the reaction which has taken place in the form of a chemical equation.

Experiment 57. Iron in Liver.—Keep a piece of liver in a potassium ferrocyanide solution for a few minutes. Then add some dilute HCl (0·5 per cent.). A faint blue colour appears. Carry out the same experiment with a piece of kidney or any other tissue. Record the results.

Experiment 58.—Apply the same test to a piece of liver from a case of pernicious anæmia, in which there is an excessive destruction of red blood corpuscles. A distinct blue colour appears. Explain the results.

NERVOUS TISSUE.

Nervous tissue differs from other tissues in being particularly rich in *lipoids*, *i.e.*, substances soluble in fat-solvents, such as ether, alcohol, chloroform, and insoluble in water. In order to extract lipoids from tissues it is best to dry the tissues first. This is done by finely mincing the tissue, sprinkling it with formalin in order to avoid putrefaction, and, after removing the bulk of the

water, driving off the rest by means of a current of hot air obtained from a fan or by placing it in an incubator at 40°. The dried nervous tissue is then treated as follows :—

Experiment 59. Separation of Lipoids of Nervous Tissue.

Cholesterin.

Place 3-5 gm. of dried tissue in a flask, and extract three times with cold acetone, which dissolves cholesterin. Decant the acetone through a filter, unite all the acetone extracts in a porcelain basin, and evaporate the acetone in a fume cupboard, by placing the basin on a hot-water bath. Scrape off the residue and label "Cholesterin."

Phosphatides (Lecithin, Kephalin).

The nervous tissue which has been freed from cholesterin by acetone extraction is now extracted with cold ether in the same way. The ether extracts are united and evaporated in the same way. Label the residue, which contains the ether-soluble phosphatides lecithin and kephalin, "Phosphatides."

Cerebrosides and Phospho-Cerebrosides (Cerebron, Protagon).

The nervous tissue which has thus been freed from cholesterin and phosphatides is now extracted with boiling alcohol or chloroform. These extracts are united and evaporated in the same way. Label residue, "Cerebrosides and Phospho-cerebrosides."

After having in this way separated three groups of

lipoids apply the following tests to each of these groups, and verify the presence or absence in each group of the substances tested for. Record your results. Since separation of the various lipoids is not complete, note whether tests are strongly positive or only faintly positive.

Experiment 60. Tests for Cholesterin.—(*a*) *Crystalline form.*—Dissolve the solid in very little hot alcohol, filter and place a drop of the alcoholic solution on a slide. Allow the alcohol to evaporate, and examine under the microscope, whether the characteristic plates of cholesterin have been formed. Note that cerebron and protagon also crystallise from alcohol, but that the form of the crystals is quite different. Sketch the crystals obtained. Add a drop of strong sulphuric acid; the edges of the crystals turn red.

(*b*) *Salkowski's test.*—Dissolve some of the solid in chloroform and apply Salkowski's test as described in Experiment 31.

Experiment 61. Test for Phosphatides.—This test depends on the presence of a phosphoric acid group in the molecule.

Incinerate a little of the material in a crucible. Cool, extract the ash with 2 or 3 c.c. of hot water and filter. To a few drops of the filtrate add about 5 c.c. of ammonium molybdate solution and 3-5 drops of pure nitric acid. Heat gently. A yellow precipitate indicates the presence of phosphatides or phospho-cerebrosides.

What is the yellow precipitate?

Experiment 62. Tests for Cerebrosides and Phospho-Cerebrosides.—These tests depend on the presence of the reducing sugar galactose in the molecule.

(*a*) *α-Naphthol test.*—Boil some of the material with water, cool and apply Molisch's test as described in Experiment 15.

If α-naphthol test is positive verify by (*b*) *Reduction test.*—Boil some of the material with a little dilute hydrochloric acid, neutralise and apply Fehling's test.

(*c*) *Presence of Phosphoric Acid.*—Test as described in the preceding experiment. A negative result together with a positive test for galactose indicates that cerebrosides only are present. If both the tests for phosphoric acid and for galactose are positive, phospho-cerebrosides are present.

Histochemistry of Lipoids (Demonstration).—Examine under a polarisation microscope: olein, lecithin, cholesterin, protagon. Note that olein is isotropous, *i.e.*, not double refracting, while lecithin, cholesterin, and protagon are anisotropous, *i.e.*, double refracting.

Examine a piece of a sciatic nerve (either fresh or fixed in formalin) under the polarisation microscope. Note that the medullary sheath is double refracting along its entire length.

Examine a piece of a degenerated sciatic nerve (about two weeks after the lesion) under the polarisation microscope. Note that the medullary sheath now shows double refracting globules of myelin alternating with non-double refracting globules of fat.

Prepare 2 per cent. chloroform solutions of olein and of the lipoids present in the myelin of nervous tissue: lecithin, cholesterin, protagon. To about 1 c.c. of each, placed in a small test tube, add one drop of 1 per cent. osmic acid solution. Note that olein and lecithin blacken rapidly, cholesterin and protagon only slowly.

Prepare thin films of these lipoids, and treat them according to Marchi's method for degenerated nerves. Proceed as follows:—Allow two drops (not more) of the chloroform solution to fall on a disc of filter paper about 1·5 cm. in diameter. Dry in incubator. Place disc

carrying a thin film of lipoid first in a 25 per cent. potassium bichromate solution (Müller's fluid) for three days at 40°, then in a mixture of two parts of Müller's fluid and one part of 1 per cent. osmic acid solution. Note that now only olein blackens.

BLOOD.

Blood is a tissue, the cellular elements of which are suspended in a fluid—the blood plasma.

COAGULATION OF BLOOD.

(Demonstration.)

Experiment 63. Formation of Clot and Serum.—Blood is allowed to flow from a blood vessel directly into a clean conical glass vessel. A blood clot is formed. Note its soft, spongy character. Allow the clot to stand for several hours without shaking; the clot contracts and expresses a clear fluid—the *blood serum*. Note that the clot adheres, as a rule, in its upper layers to the walls of the glass vessel. If the process has been carried out carefully, the serum will not show the faintest trace of a red colour. The appearance of such a colour in the serum indicates that hæmolysis of the red corpuscles has taken place (see Exp. 70).

Wash a part of the clot in running water. The red blood corpuscles which are entangled in the clot are broken up, as the deep red colour of the wash-water indicates. If the washing is continued sufficiently long, a yellowish-white stringy mass remains, which gives Millon's test and the xanthoproteic test for proteins. This is *fibrin*.

Experiment 64. Defibrination of Blood. Preparation of Serum and of a Suspension of Blood Corpuscles.—Blood

is allowed to flow from a blood vessel into a dish, and stirred there gently but continuously with a wooden stick or any other object presenting a rough surface (bundle of feathers). A stringy mass of fibrin, coloured red by entangled blood corpuscles, gradually collects at the end of the stick. After five to ten minutes' stirring, the stick, with the adherent stringy mass, which is much denser and harder than the clot found in the previous experiment, is withdrawn. The remaining fluid is *defibrinated blood.* It resembles blood in its external appearance, but differs from it in the fact that all the fibrin, and fibrin-yielding substances, have been removed from it. On standing, or more rapidly on centrifugalisation, defibrinated blood separates into *two* layers: a layer of *red blood corpuscles* and a supernatant layer of a clear fluid, *the serum,* identical in composition with the serum expressed from the clot in the previous experiment. The serum can be removed by means of a pipette.

The blood corpuscles are freed from traces of adherent serum by suspending them in 0·9 per cent. saline solution, centrifugalising the solution, and removing the wash-fluid until it ceases to give the biuret test for proteins. 5 c.c. of washed blood corpuscles are then added to 95 c.c. of 0·9 per cent. NaCl solution, and the mixture shaken. It represents a 5 per cent. suspension of red blood corpuscles. Label "5 per cent. r.b.c."

By this method only one constituent of circulating blood can be prepared unchanged—the red blood corpuscles. The fluid obtained in this experiment, the serum, differs from the fluid in which the cellular elements of the circulating blood are suspended, the plasma. In order to study the constituents of circulating blood, it is necessary to prevent the coagulation of blood. The following experiment represents the methods most frequently used for that purpose.

Experiment 65. Constituents of Circulating Blood (Cellular Elements and Plasma). Methods of Preventing the Coagulation of Blood.—

(*a*) Coat an artery canula and several glass vessels with a mixture of two parts of hard paraffin, two parts of soft paraffin, and one part of stearin.

Insert the canula into the artery without touching the tissues surrounding the artery. Allow blood to flow into the paraffined vessel. The blood does not clot, or if the experiment has not been entirely successful, clots very much more slowly than in Experiment 63.

(*b*) Place in a shallow wide-mouthed bottle 10 c.c. of a 1 per cent. solution of potassium oxalate in 0·7 per cent. NaCl solution. Allow blood to flow into this solution from a blood vessel, so that the blood does not touch either the tissues of the wound or the walls of the glass vessel. When the mixture of blood and oxalate solution in the bottle has reached a level of 50 c.c., previously marked on the vessel, the bottle is withdrawn, and a fresh one may be substituted. The oxalate, which has then a concentration of 0·2 per cent., has precipitated all the calcium salts of the blood. The blood does not clot.

If blood which has been prevented from coagulation by either of these methods is allowed to stand, or, more rapidly, if it is centrifugalised, it separates into *three* layers:—1. A layer of *red blood corpuscles* at the bottom of the tube. 2. A narrow greyish layer consisting mainly of *blood platelets* and some *leucocytes*. 3. A fluid, the *blood plasma*, which occupies the upper half or two-thirds of the tube. Note that the plasma is fairly clear in its upper layers, but slightly turbid in the lower layer, where it adjoins the layer of platelets. Note further that in standing the platelets agglutinate and form a coherent cake—a

"white thrombus." Note that there is an essential difference between a "white thrombus" and a blood clot.

Experiment 66.—Conditions Inducing the Coagulation of Blood.—Receive blood in paraffined vessels as described in the previous experiment. Pour some blood from a paraffined vessel into a glass vessel: coagulation sets in. To some blood contained in a paraffined vessel add a piece of a feather: coagulation sets in. The experiment shows that contact with objects which are wetted by blood (*e.g.*, glass and feather) induces coagulation; contact with objects not wetted by blood (*e.g.*, paraffin) does not do so. Blood does not wet the endothelial lining of blood vessels.

Experiment 67. Factors Concerned in the Coagulation of Blood. Separation of Plasma and Blood Platelets.—From oxalated blood, obtained as described in Experiment 65 (*b*), isolate the following constituents.

1. **Plasma and Platelets.**—Centrifugalise oxalated blood; remove the plasma by means of a pipette. Owing to its contamination with blood platelets the plasma is slightly turbid. Label "Plasma and Platelets."

2. **Plasma.**—Filter some of this mixture of plasma and platelets through a porous porcelain filter (a "Berkefeld" filter), in order to remove all cellular elements. The filtrate is quite clear. Label "Plasma filtered."

3 **Extract of Blood Platelets.**—Remove by means of a pipette parts of the greyish intermediate layer of platelets from centrifugalised oxalate blood. Suspend in 0·9 per cent. saline solution. Centrifugalise until the platelets are deposited at the bottom of the tube. Decant supernatant fluid. Treat the sediment with about 5 c.c. of distilled water, which breaks up the platelets. Label "Platelet Extract."

Prepare a 1 per cent. solution of calcium chloride. Label small test tubes A, B, C, D. Charge with the various solutions as given below, and place in water-bath at 35°.

Tube.	Contents.	Result.
A	1 c.c. oxalate blood + 0·2 c.c. $CaCl_2$ - - -	Clot.
B	1 c.c. oxalate plasma and platelets + 0·2 c.c. $CaCl_2$ - - - - - - - - -	Clot.
C	1 c.c. oxalate plasma filtered + 0·2 c.c. $CaCl_2$ -	No clot.
D	1 c.c. oxalate plasma filtered + 0·2 c.c. $CaCl_2$ + 0·2 c.c. platelet extract - - - - -	Clot.

These results show—

1. That calcium salts are necessary for coagulation (tubes A and B).

2. That red blood corpuscles are not necessary (tube B).

3. That platelets are necessary (tubes B and C).

4. That the function of the platelets can be replaced by a watery extract of platelets (tube D).

Summary.—In the reaction which leads to the formation of a blood clot, the following constituents are concerned: (1) Blood platelets; (2) soluble calcium salts, which are present in circulating plasma; (3) yet another constituent of the plasma.

Experiment 68. Separation from Plasma of Mother Substance of Fibrin: "Fibrinogen." Difference in Composition between Plasma and Serum.—Prepare "oxalate plasma filtered" as described in the previous experiment. Prepare fresh serum by any of the methods described above.

To 10 c.c. of filtered plasma add an equal volume of a saturated NaCl solution, so that the salt concentration of the mixture is that of a half-saturated NaCl solution. A white precipitate appears of one of the protein substance of plasma, namely *fibrinogen.*

Repeat the same experiment with serum. No precipitate

appears. (For further investigation of proteins of serums see Experiments 85, 86.)

Separate the fibrinogen precipitate from the plasma by centrifugalising, decant the supernatant fluid. Wash the precipitate with a half-saturated solution of NaCl (in which it is insoluble), and dissolve it in 10 c.c. of a 0·9 per cent. NaCl solution. Label "Fibrinogen Solution."

With a little of the fibrinogen solution perform biuret test and heat coagulation test for proteins. Both are positive.

Label four test tubes E, F, G, H; charge with the various solutions, and place in water-bath kept at 35°.

Tube.	Contents of Tubes.	Result.
E	1 c.c. fibrinogen solution + 0·2 c.c. $CaCl_2$ - -	No clot.
F	1 c.c. fibrinogen solution + 0·2 c.c. $CaCl_2$ + 0·2 c.c. platelet extract - - - - -	Clot.
G	1 c.c. fibrinogen solution + 0·2 c.c. $CaCl_2$ + 0·2 c.c. serum - - - - - - -	Clot.
H	1 c.c. fibrinogen solution + 0·2 c.c. $CaCl_2$ + 0·2 c.c. plasma, filtered - - - - -	No clot.

These experiments show :—

1. That the protein precipitated from plasma by half saturation with NaCl is the third constituent concerned in the formation of a blood clot.

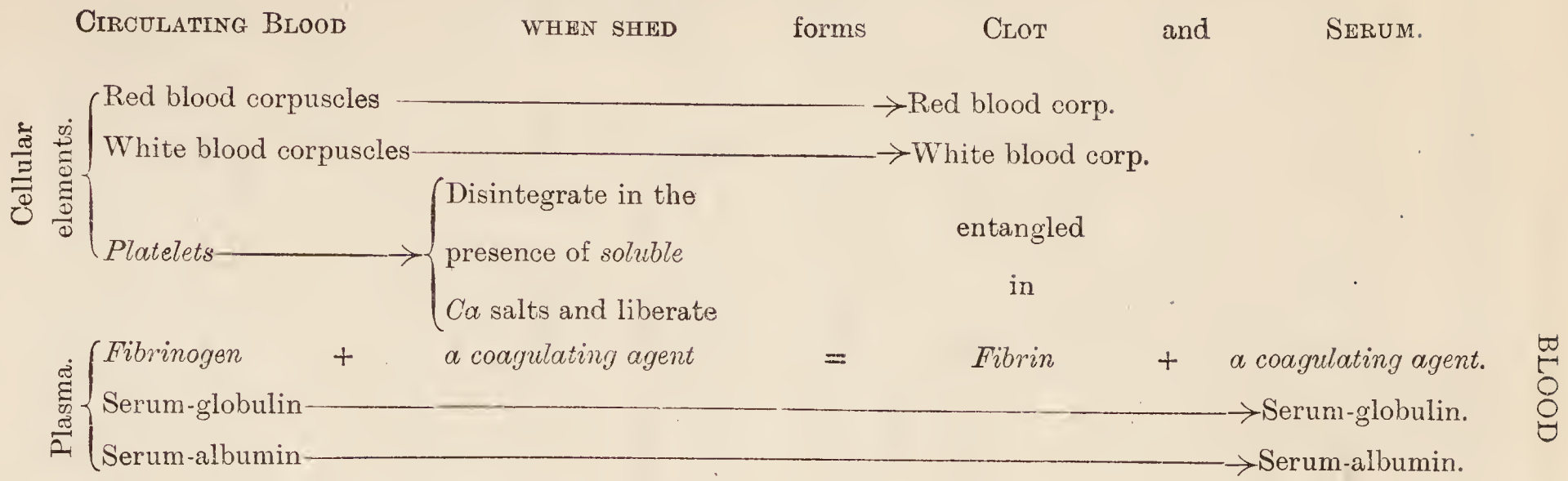
2. That this protein when acted upon in the presence of soluble calcium salts by the coagulating agent present in the platelets gives rise to fibrin (tube F).

3. That this protein (fibrinogen) is absent from serum.

4. That the coagulating agent remains active after the clot has been formed, *i.e.*, it is present in serum (tube G). It is absent from plasma (tube H).

From these experiments the main facts of the process of blood coagulation can be tabulated as follows :—

THE COAGULATION OF BLOOD.



LAWS OF BLOOD COAGULATION.

FIRST LAW.—*No blood coagulation without disintegration of platelets.*

SECOND LAW.—*Everything that facilitates the disintegration of platelets, facilitates the coagulation of blood.*

Everything that retards or prevents the disintegration of platelets, retards or prevents the coagulation of blood.

HÆMOLYSIS. RESPIRATORY FUNCTIONS OF BLOOD.

For Experiments 69-83 defibrinated blood is used.

Experiment 69. Reaction of Blood.—Apply a drop of defibrinated blood to glazed litmus paper. Allow it to remain for a minute and then wash off with water. Why is it necessary to use glazed litmus paper?

Determine in the same way the reaction of freshly drawn blood obtained by pricking the finger.

What bearing has the reaction of the blood on its respiratory function?

Experiment 70. Laking of Blood. Hæmolysis.—Label three test tubes A, B, and C. Place in each 1 c.c. (= twenty drops) of defibrinated blood. To B add 5 c.c. of water, to C two or three drops of ether. Then fill all three test tubes with 0·9 per cent. NaCl solution, and compare.

In A the solution is opaque, because the blood corpuscles have remained intact; in B and C it is transparent, because the blood corpuscles have been laked. Explain the results.

Experiment 71. Oxidising Ferment of Blood, "Guaiac Test."—Dilute 5 drops of blood with about 10 c.c. of water, add some hydrogen peroxide and float on the surface of the fluid two drops of alcoholic tincture of guaiac resin so that the latter forms a resinous ring above the fluid. A blue colour gradually develops in the resinous ring. Explain the reaction.

The reaction is not specific for blood, but given by many other animal and vegetable tissues and secretions such as milk. Blood differs from the other tissues, however, in so far as the reaction is given by blood even after it has been boiled, *i.e.*, after the ferment has been destroyed.

Repeat the experiment with diluted blood which has first been boiled. A positive result is obtained. In blood, therefore, the reaction is due not only to an oxidising ferment but also to some thermostable chemical constituent, probably the blood pigment.

Sensitiveness of the Guaiac test.—The test is very sensitive for blood. Determine the greatest dilution of blood with which the test can still be obtained.

Experiment 72. Determination of Oxygen-Capacity of Blood.—The oxygen-capacity is the maximum amount of oxygen that can be held by blood. It is dependent on the amount of hæmoglobin present.

The apparatus consists of a burette, which is inverted in a tall jar filled with cold water, and is connected by tubing to a bottle containing a small test tube. The bottle is placed in a vessel (water-bath) filled with cold water.

Place some blood in a porcelain dish and stir vigorously with a glass rod, until it becomes completely saturated with oxygen. By means of a pipette run exactly 20 c.c. of blood into the bottle. Add to it 30 c.c. of dilute ammonia (1 : 500). The water lakes the blood; the ammonia is added in order to prevent carbonic acid gas being given off. In the small test tube place 4 c.c. of saturated potassium ferricyanide solution. Put the test tube into the bottle, taking care that the ferricyanide is not spilt. Close the bottle with the stopper, and put it into cold water.

Test whether the apparatus is air-tight by raising the burette; if there is no leakage the column of water in the burette remains standing at a higher level than the water in the jar.

Allow the apparatus to stand for five minutes, so that all parts of it acquire the same temperature. Then open for a few seconds the clip on the tubing, which connects

the inside of the bottle to the outer air. The pressure inside the apparatus is now the same as the pressure outside. Close the clip. The level of the water inside the burette is then at the same height as the level of the water in the jar. Read off the height in the burette with the water inside and outside at the same level.

Now tilt the bottle so that the ferricyanide solution is upset, and shake gently as long as gas is evolved. When no more gas is evolved, replace the bottle in the water and wait five minutes. What reaction has taken place?

Read the burette in the same way as before, *i.e.*, with the water inside and outside at the same level. (Why is this necessary?) The difference in readings gives the amount of oxygen held by 20 c.c. of defibrinated blood. Record the result.

N.B.—In measuring gases, as in this experiment, the temperature must be kept as constant as possible. (Why?) The apparatus should therefore be touched with the hands as little as possible, and no source of heat (gas flame, bunsen, etc.) must be near it.

CHEMISTRY OF BLOOD PIGMENT.

ABSORPTION SPECTRA OF HÆMOGLOBIN AND ITS DERIVATIVES.

Spectroscopic Examination of Blood Pigments.

Solutions of hæmoglobin and its derivatives give characteristic absorption spectra, which can be examined with a spectroscope.

What is an absorption-spectrum?

Examine the spectroscopes provided. Note that the width of the slit at one end can be adjusted,[1] and that the

[1] In some spectroscopes the width of the slit is fixed.

spectrum can be focussed by adjusting the eyepiece. The sharpness of the spectrum is dependent upon the width of the slit, and is the sharper the narrower the slit.

Experiment 73. Solar Spectrum.—Direct the spectroscope to the sky. If the slit and the eyepiece are properly adjusted, fine vertical dark lines can be seen in the spectrum. These are Fraunhofer's lines. How are they produced?

The lines are designated by letters. Line B is in the red, line D in the yellow, line E in the green, line F in the blue. Their position in the spectrum is constant, and they can be used to locate certain parts of the spectrum.

Experiment 74. Spectrum of White Light.—Now direct the spectroscope to a luminous gas flame (or to any other source of artificial light). The spectrum is still visible, but it does not show Fraunhofer's lines.

Explain why Fraunhofer's lines are absent.

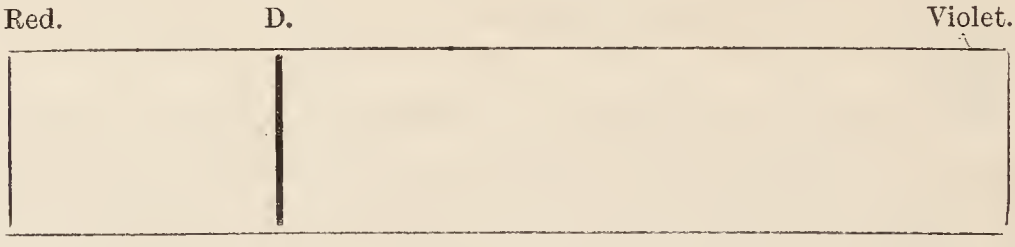
Experiment 75. Spectrum of Sodium in White Light.—Introduce into the gas flame an asbestos stick soaked in sodium chloride, so that a sodium flame is produced. A bright yellow band is seen in the yellow part of the spectrum, and if the slit of the spectroscope is sufficiently narrow this yellow band can be seen to consist of two sharply defined narrow lines. These lines are in the same position as the black line D of the solar spectrum.

Most of the absorption spectra of hæmoglobin and its derivatives lie in the neighbourhood of the D line, and their position with reference to the D line can be easily recognised by using a sodium flame as described in Experiment 75.

NOTE.—*By convention the spectrum is usually arranged in such a way that the red end is on the observer's left-hand side.*

In observing the spectrum of any fluid the degree of dilution is of importance.

Record diagrammatically the absorption spectra of oxyhæmoglobin, hæmoglobin, carboxyhæmoglobin, methæmoglobin, and hæmochromogen on charts, using the following chart as a model:—



Experiment 76. Oxyhæmoglobin.—Take some defibrinated blood in a test tube and run in water slowly from the tap, holding the tube obliquely under the end of the pipe; allow the water to continue running after the tube is full. Thus one obtains a solution of oxyhæmoglobin diluted in such a way that the upper part of the tube contains almost pure water, the lower part a very concentrated solution of the pigment, and the middle part all gradations between the two. The corpuscles are of course laked.

Adjust the spectroscope as described above; place the upper end of the test tube, which must be quite dry, against the slit, holding the tube by the lower end with the left hand.

On looking through the spectroscope probably no bands will be seen. Gradually raise the tube so as to bring a stronger solution of oxyhæmoglobin in front of the slit. Two bands will appear, one narrower than the other and nearer the red end of the spectrum. In stronger and stronger solutions these two bands fuse into one, and broaden out so as to obscure the whole spectrum.

Prepare a solution of oxyhæmoglobin so that the two bands can be clearly seen. Direct the spectroscope towards a sodium flame. It will be seen that both bands

lie to the right of the D line, the left band lying close to the D line.

Experiment 77. Hæmoglobin. (Reduced hæmoglobin). —Reduce the solution of oxyhæmoglobin prepared in Experiment 76, which shows the two bands of oxyhæmoglobin distinctly. Reduction is performed by adding a reducing agent —one drop of a freshly prepared 10 per cent. solution of sodium hydrosulphite ($Na_2S_2O_4$), or five drops of Stokes' solution, made alkaline with a few drops of ammonia (see below), or five drops of ammonium sulphide. If ammonium sulphide is used for reduction, the tube must be warmed gently to about 50°. Note that the scarlet-red colour of oxyhæmoglobin (arterial blood) gives place to the bluish-red colour of hæmoglobin (venous blood). Examine spectroscopically. The spectrum now shows a single broad band, which overlaps the space enclosed by the two bands of oxyhæmoglobin, and is fainter than either.

Locate its position with reference to the D line. It is immediately to the right of the D line.

Preparation of Stokes' Solution. — Dissolve 2 gm. of tartaric acid in a little water. In another small quantity of water dissolve 2 gm. of ferrous sulphate. Mix the two solutions and make the mixture up to 100 c.c. with water. Fill into bottle labelled "Stokes' solution." Before use a few drops of ammonia are added, so that the solution is just alkaline.

Experiment 78.—Close with the finger the test tube containing hæmoglobin as prepared in Experiment 77, and shake vigorously for two to three minutes. Note that the colour of the solution changes from bluish red to scarlet red. Examine at once with the spectroscope; the two bands of oxyhæmoglobin have reappeared. (Why?) Allow the test tube to stand for two or three minutes; reduction takes place again owing to the presence of

excess of the reducing agent, and the single band of hæmoglobin is again seen in the spectrum.

Experiment 79. Carboxyhæmoglobin.—Prepare some CO-hæmoglobin by filling a test tube with coal gas, and adding some diluted blood; close the tube with the thumb, and shake. Notice the pink colour. This pink colour persists even if the pigment is much diluted with water. (Contrast with oxyhæmoglobin, which becomes yellow if much diluted with water.)

Examine CO-hæmoglobin spectroscopically, diluting the solution until two absorption bands are distinctly visible. The two bands are apparently similar to those of oxyhæmoglobin. Verify by locating the position of the bands with reference to the D line, using a sodium flame. The two bands have almost the same position immediately to the right of the D line.

Carboxyhæmoglobin is not acted upon by reducing agents: Distinction from oxyhæmoglobin. — Add five drops of ammonium sulphide. No change takes place: the bands persist. Add five drops of Stokes' solution, made alkaline with ammonia. Same result.

Experiment 80. Methæmoglobin.—To 5 c.c. of water add four drops of blood. To the strong solution of oxyhæmoglobin thus prepared add a few drops of potassium ferricyanide. The solution becomes brown, and the spectrum shows a distinct band in the red. Locate by means of the D line, using a sodium flame: the band is to the left of the D line. This is the characteristic band.

With this concentrated solution there is marked absorption in the blue end of the spectrum. Dilute with an equal bulk of water; two faint bands appear in the green.

Add to the dilute solution of methæmoglobin a few drops of ammonium sulphide and warm gently. The colour

changes to red. Examine immediately with the spectroscope. At first the spectrum of oxyhæmoglobin appears, then that of reduced hæmoglobin.

What is the difference between methæmoglobin and oxyhæmoglobin?

Experiment 81. Alkaline Hæmatin. Hæmochromogen (or reduced alkaline hæmatin).—Prepare alkaline hæmatin by warming some diluted blood with caustic soda. Warm gently at first, then heat to near boiling point, but do not allow to boil. The colour changes to brown. Cool. The spectrum is indistinct. It shows a faint band in the red to the left of the D line.

Now add a few drops of sodium hydrosulphite or of ammonium sulphide; the very distinct spectrum of hæmochromogen appears. It consists of two bands in the green. Locate by means of the D line, using a sodium flame. The two bands are to the right of the D line, as in the case of the spectra of oxyhæmoglobin or CO-hæmoglobin, from which the bands of hæmochromogen differ by lying further towards the blue end of the spectrum. There is a distinct space between the left band of hæmochromogen and the D line, while in the case of oxyhæmoglobin and CO-hæmoglobin the left band abuts against the D line.

If the solution of hæmochromogen is diluted, the left band persists longer, being the stronger band, so that in very weak solutions only this band is seen.

Experiment 82.—The formation of hæmochromogen is a very delicate test for blood. Dilute blood until the spectrum of oxyhæmoglobin cannot be readily seen. Convert this very dilute solution of oxyhæmoglobin into hæmochromogen as detailed in Experiment 81. The formation of hæmochromogen can be detected by the presence of the left band.

Experiment 83. Hæmatoporphyrin.—Place 3 c.c. of pure concentrated sulphuric acid in a test tube, and allow one drop of defibrinated blood to fall into it. The fluid shows a purple colour. Examine spectroscopically. Dilute, if necessary, with concentrated sulphuric acid. The spectrum shows two bands, one which is near the red end of the spectrum being much narrower than the other. Locate their position with reference to the D line. The narrower band is immediately to the left of the D line, so that the D line is enclosed by the two bands.

Why is concentrated sulphuric acid necessary for the formation of this derivative?

What is the essential difference between the chemical composition of hæmatoporphyrin and that of the other hæmoglobin derivatives?

CHEMISTRY OF BLOOD-SERUM.

Experiment 84. Reaction and Specific Gravity of Serum.—Test the reaction of serum against litmus.

Determine the specific weight by means of a hydrometer. Wipe the instrument clean, and float it in the centre of the cylinder containing the serum, taking care that it does not touch the sides of the vessel. Place the eye level with the true surface of the serum (not the top of the meniscus around the shaft of the hydrometer), and read the division of the scale. Record your result.

Experiment 85. Proteins of Serum. Separation of Globulin and Albumin.—Apply to serum the protein tests. (Exp. 33, 34.) Note that they are all very strongly positive. Separate the proteins present in the serum by the "salting out" method. Proceed as follows:—

Proteins of Blood-Serum.

To 10 c.c. of the serum add an equal volume of a

saturated solution of ammonium sulphate, thus obtaining a half-saturated solution. Filter off the precipitate of *serum-globulin* which appears, and remove the filtrate, which contains *serum-albumin*. Wash the precipitate two or three times with a half-saturated ammonium sulphate solution, and dissolve it, together with the ammonium sulphate which adheres to it, in about 20 c.c. of water. This yields a clear solution of a globulin in a dilute salt (ammonium sulphate) solution. Label it "*Serum-globulin.*"

To the filtrate from the serum-globulin add solid ammonium sulphate until the solution is saturated, keeping the solution at a temperature of 20° to 30° C. in order to facilitate the dissolving of the ammonium sulphate. Higher temperatures must be avoided. Why? When the solution is saturated a precipitate of albumin is formed. Note the difference between the dead-white, heavy crystals of ammonium sulphate lying at the bottom of the vessel and the yellowish-white, light, flocculent precipitate of albumin which is suspended in the liquid. Filter. Wash the precipitate on the filter with a saturated solution of ammonium sulphate, and dissolve it in about 20 c.c. water. A clear solution of an albumin is obtained. Label it "*Serum-albumin.*"

Solubilities of Albumin and Globulin.

Experiment 86. Solubility of Albumin and Globulin in Water and in Concentrated Salt Solutions.—Add a few drops of the globulin solution to a large volume of distilled water in a beaker. A slight, cloudy precipitate is formed.

Do the same with the albumin solution. No precipitate is formed.

Saturate some of the globulin solution with solid sodium chloride. The globulin is precipitated.

Saturate some of the albumin solution with solid sodium chloride. The albumin is not precipitated.

Record in tabular form the solubilities of albumin and globulin according to the following table:—

Solvent.	Albumin.	Globulin.
Distilled water - - - - -		
Dilute NaCl solution - - - -		
Saturated NaCl solution - - -		
Half-saturated $(NH_4)_2SO_4$ solution -		
Full-saturated $(NH_4)_2SO_4$ solution -		

Apply to the albumin and globulin solutions the heat coagulation test. Note that both albumin and globulin are coagulable by heat.

Apply the colour tests for proteins. Note that the xanthoproteic test is quite distinct, but that the biuret test may be negative. This is due to the fact that the albumins and globulins have been separated by means of ammonium sulphate, and that ammonium salts interfere with the biuret test.

Study the effect of the presence of an ammonium salt on the biuret test in the following way: Apply the test to (*a*) water; (*b*) water to which some ammonium sulphate has been added; (*c*) serum; (*d*) serum to which some ammonium sulphate has been added. The following colours will be obtained:—

(*a*) Water - - - faint blue.
(*b*) Water + $(NH_4)_2SO_4$ - deep blue.
(*c*) Serum - - - violet.
(*d*) Serum + $(NH_4)_2SO_4$ - deep blue.

Only (*c*) gives the characteristic violet colour which indicates the presence of proteins. Ammonium salts therefore interfere with the test. This must be borne

in mind when the test is applied after having used ammonium salts for the separation of proteins. The difficulty can be overcome by using in such a case a very large amount of caustic soda before adding the copper sulphate. Try this with some serum to which ammonium sulphate has been added.

Method of Removal of Proteins by Heat Coagulation.

Experiment 87. Serum Constituents other than Proteins. Method of removal of heat-coagulable Proteins.—In order to determine the presence of substances other than proteins in tissue fluids or tissue extracts it is necessary to remove completely the proteins, as their presence interferes with the tests used for other tissue constituents (see also Exp. 39). If the proteins are coagulable by heat, as is the case with serum, the proteins are best removed by heat coagulation. It is important to carry out this process carefully so that all the proteins are completely precipitated. Proceed in the following manner: Dilute 20 c.c. of serum with 100 c.c. of water; heat the neutral solution to boiling point, stirring constantly. Make faintly acid with a few drops of dilute acetic acid, and filter. If the heat coagulation has been carried out correctly (see Exp. 33), the filtrate will not give the biuret test, *i.e.*, it will be free from proteins.

Concentrate the filtrate to about 20 c.c., and apply to it the following tests:—

(*a*) Test for chlorides with silver nitrate and nitric acid.

(*b*) Test for phosphates with ammonium molybdate and nitric acid.

(*c*) Test for sulphates with barium chloride and hydrochloric acid.

(*d*) Test for a reducing sugar with Fehling's or Trommer's test.

Record your results.

METHOD OF CHEMICAL EXAMINATION OF TISSUES.

From the foregoing experiments it follows that the three main groups of organic substances which may be present in tissues and tissue fluids, namely, (1) *proteins*; (2) *carbohydrates*, and (3) *fats and lipoids*, can be separated from each other by extraction with different solvents. The various substances of which each group is composed can then be recognised by their specific reactions and properties.

The following general rules for the chemical examination of tissues and tissue fluids can be deduced from the work hitherto performed:—

The organs must be minced.

I. *Proteins.*

(*a*) Extraction with dilute salt solution dissolves—

1. *Simple Proteins* (*Albumins, Globulins*).—They give the tests for proteins. They are coagulated by heat. In order to ascertain whether an albumin or a globulin is present, determine the solubility in distilled water, in half-saturated and in fully-saturated ammonium sulphate solution. If both are present they can be separated by their different solubility in ammonium sulphate solution.

2. *Mucin.*—It gives all the tests for proteins. It is precipitated by acetic acid, and is insoluble in excess of this acid (see Exp. 88). On boiling with dilute HCl it yields a reducing substance, glucosamin. This last test can be carried out only if sufficient material is obtained.

The saline extract will also contain carbohydrates and extractives (see below).

(*b*) Extraction with 1 per cent. sodium carbonate or sodium hydrate solution dissolves *Nucleoprotein.* It gives all the protein tests. It is precipitated by dilute acetic acid, but soluble in excess of this acid. This alkaline extract will also contain *Mucin* (see above).

II. *Carbohydrates.*—Extraction with hot water will dissolve all the carbohydrates: *Starch, glycogen, dextrin, sugars.* These may be recognised by their tests. The extract may contain a small amount of protein substances (test by means of biuret test); these must be removed by precipitation or heat coagulation before the tests for carbohydrates can be applied. The watery extracts also contain *inorganic salts* and *organic extractives*, such as *urea, creatin, purin-bases*, etc. The commercial meat extracts consist mainly of such organic extractives.

III. *Fats and Lipoids.*—The tissue is dried and extracted successively—

(*a*) With acetone, which extracts mainly *fats* and *cholesterin.*

(*b*) With ether, which extracts mainly *phosphatides.*

(*c*) With chloroform (or hot alcohol), which extracts lastly *cerebrosides, phospho-cerebrosides, cholesterin esters.*

If one wishes to remove all fats and lipoids without separating them, extraction with chloroform, which dissolves all lipoids, is applied at once.

The method is essentially the same if tissue fluids, exudates, etc., are to be examined, except that the tests for proteins are applied directly to the fluid. If any proteins are present, they must be removed before examining for carbohydrates. Fat is extracted by shaking the fluid with

ether, which, after allowing the two fluids to separate, is removed by means of a pipette, or by decantation.

If contents of the alimentary canal are to be examined, products of protein digestion may be present, and must be tested for by the method described under Digestion (see below, Exp. 109).

DIGESTION.

SALIVA.

The function of the saliva is not only a digestive one. It plays an important part in assisting to prevent caries of the teeth by removing and dissolving remnants of food from the teeth. Bacterial fermentation of remnants of food in the mouth forms alkaline substances from proteins, acid substances from carbohydrates. The acid substances erode the teeth. Hence the association of dental caries with dietetic errors, such as the excessive consumption of sticky carbohydrate foods which the saliva cannot easily remove from the teeth.

Experiment 88.—Collect about 10 c.c. of saliva in a beaker. Test its reaction to litmus. Record.

Mucin.

Add dilute acetic acid to some saliva in a test tube. A stringy precipitate of mucin is formed. It is insoluble in excess of acetic acid.

Prepare some dilute saliva as described in Experiment 18. With this dilute saliva carry out the following experiment:—

Digestion by Saliva.

Experiment 89. Prove that Saliva contains an Amylolytic (Diastatic Ferment: Ptyalin.—To 5 c.c. of starch solution (1 per cent.) in a test tube add 5 c.c. of dilute saliva. Mix by shaking and place in a water-bath

kept at 37° to 40°. Place a series of drops of dilute iodine solution on a porcelain slab. Every half minute remove a drop of the salivary digest by means of a glass rod, and apply it to one of the iodine drops. At first a blue colour will be produced, then a reddish-violet colour, then a light brown colour, finally no colour will appear. (State what chemical changes are indicated by the changes in colour.) The point when first a colour fails to appear is called the "achromic point"; the time necessary to reach the achromic point is called the "chromic period." The determination of the chromic period is a means of estimating quantitatively the activity of the ferment, provided that a starch solution of known and constant concentration is used. State the chromic period of the saliva used for this experiment. After the achromic point has been reached, apply Trommer's or Fehling's test to the salivary digest. Reduction occurs. Which sugar is present?

Properties of Ptyalin.

Experiment 90. Prove that Ptyalin does not act in an Acid Medium.—To 5 c.c. of dilute saliva add five drops of very dilute HCl (0·4 per cent.), and mix this with 5 c.c. of starch solution. Then determine the "chromic period" as detailed above. Record your result. What bearing has the effect of dilute HCl on the digestion of carbohydrates in the stomach?

Experiment 91. Prove that the Ferment Ptyalin is destroyed by Heat.—Heat 5 c.c. of dilute saliva to boiling point, cool, and mix with 5 c.c. of starch solution. Determine the "chromic period" as detailed above Record your results.

Experiment 92. Prove that the Activity of the Ferment Ptyalin is arrested or retarded by Cold.—Mix

5 c.c. of dilute saliva with 5 c.c. of starch solution, and place the mixture into a beaker filled with cold water. Determine the "chromic period."

GASTRIC DIGESTION.

GASTRIC JUICE.

An extract of the gastric mucous membrane is supplied.

Experiment 93.—Test the reaction to litmus. It is acid. The acid which is present in gastric juice is hydrochloric acid.

The following Experiments (94 to 101) should be carried out at the same time.

Properties of Pepsin.

Experiment 94. Prove that the gastric juice contains a proteolytic ferment: Pepsin.—Place small shreds of fibrin in three or four different test tubes, labelled A. Add about 5 c.c. of the gastric juice to each, and place the test tubes in a water-bath which is kept between 35° and 40°. The fibrin swells up, becomes transparent, and dissolves after half an hour or an hour. The time will be longer if more than a small shred of fibrin is placed in each tube. Reserve the contents of test tubes for Experiment 109.

Experiment 95. Prove that the action of this ferment is dependent upon the presence of a trace of free HCl (about 0·2 per cent.).—Place a small shred of fibrin in a test tube, labelled B, and add about 5 c.c. of gastric juice, which has previously been neutralised by adding carefully, drop by drop, dilute NaOH until the solution is neutral to litmus. Place in water-bath. The fibrin remains unaltered.

Experiment 96. Prove that HCl alone cannot digest

fibrin.—Place a small shred of fibrin in a test tube, labelled C, and add about 5 c.c. of dilute HCl of the same concentration (0·2 per cent.) as the acid present in the gastric juice. Place in water-bath. The fibrin swells up, becomes transparent, but does not dissolve. This can be verified by testing the filtered fluid for the presence of protein by means of the biuret or the xanthoproteic test.

Experiment 97. Prove that the ferment is destroyed by heat.—Place a small shred of fibrin in a test tube, labelled D, and add about 5 c.c. of gastric juice which has been boiled and then cooled. Place in water-bath. The fibrin swells up, becomes transparent, but does not dissolve.

Experiment 98. Prove that at a low temperature the activity of the ferment pepsin (in the presence of HCl) is arrested.—Place a small shred of fibrin in a test tube, labelled E, add about 5 c.c. of the gastric juice, and place the test tube in a beaker filled with cold water. The fibrin does not dissolve.

Experiment 99. Prove that this result is not due to destruction of the ferment, as in Experiment 97, but to the inactivity of the pepsin at a low temperature.—Describe the experiment and record the result.

Experiment 100. Prove that in the process of peptic digestion hydrochloric acid combines with the protein.—Place about 5 c.c. of gastric juice in a test tube, labelled F, and add a large shred of fibrin. Place test tube in water-bath, and allow to digest for about half an hour. Then add one drop of Töpfer's reagent, which will give a brownish-pink colour. Compare this colour with the deep pink colour obtained on adding one drop of Töpfer's reagent to some gastric juice in which no protein has been digested.

The difference in colour is due to the fact that in the latter case the gastric juice contains all the HCl as "free

HCl," while the gastric juice in which fibrin has been digested contains part of its HCl as "combined HCl" (*i.e.*, in combination with protein).

Töpfer's reagent, like Günzburg's reagent, is sensitive only to free HCl. If no free acid is present a yellow colour is obtained. (It does not, however, distinguish between lactic acid and hydrochloric acid, as Günzburg's reagent does; see Experiment 104.)

Experiment 101. Prove that the gastric juice contains Rennin.—Repeat Experiments 47 and 48 with gastric juice. Determine how the activity of rennin is affected by acting (*a*) in a neutral medium; (*b*) in a faintly acid medium; (*c*) in a faintly alkaline medium; (*d*) by boiling; (*e*) by cold.

GASTRIC CONTENTS.

Acids in Gastric Contents.

1. Acids.—Gastric contents have, as a rule, an acid reaction. This may be due either to hydrochloric acid, which is secreted by the gastric mucous membrane, or to organic acids, such as lactic acid, butyric acid, etc., which have been formed by the decomposition of the food in the stomach. The presence of butyric acid can be detected by the characteristic smell. Special tests are necessary in order to distinguish between hydrochloric acid and lactic acid.

The following tests should be carried out with the solutions of 0·3 per cent. HCl, and of 0·3 per cent. lactic acid, which are supplied. These acids can be distinguished by the application of Günzburg's reagent (see Exp. 104). They also differ in their solubility in ether in which lactic acid dissolves (see Exp. 107).

Experiment 102.—To 10 c.c. of water in a test tube add two drops of caustic soda and one drop of phenolphthalein. A dark pink colour is produced, indicating that the solution is alkaline. Divide the solution into two equal parts, and add to the one 5 c.c. of dilute HCl, to the other 5 c.c. of dilute lactic acid. The solution becomes colourless in each case.

Experiment 103. Congo-red.—Apply a drop of each acid to paper stained with congo-red. A blue colour is produced. Note the difference in colour produced by the two acids. Allow the congo-red paper, which has thus been acted upon, to dry. Place the paper in a dry test tube and extract with ether. The blue colour produced by lactic acid disappears from the paper, which turns red again. Why? The blue colour produced by HCl remains.

Experiment 104. Günzburg's Test for Free Hydrochloric Acid.—Evaporate four or five drops of Günzburg's reagent (which consists of an alcoholic solution of phloroglucin and vanillin) on a lid of a porcelain crucible over a small flame until the mixture just begins to become dry. Charring must be avoided. A brown residue remains.

To four or five drops of Günzburg's reagent add about ten drops of hydrochloric acid, and evaporate to dryness in the same way. A carmine red colour appears.

To four or five drops of Günzburg's reagent add about ten drops of lactic acid, and evaporate to dryness in the same way. A brown residue remains.

Günzburg's test distinguishes not only between hydrochloric acid and lactic acid, but also between "free hydrochloric acid" and "combined hydrochloric acid." This important test indicates minute traces of free HCl.

Experiment 105. Uffelmann's Test for Lactic Acid.—To 10 c.c. of 2 per cent. carbolic acid add one drop of ferric

chloride. A blue colour appears. Add to this a few drops of lactic acid: the blue colour is replaced by a distinct yellow. Carry out the same test with dilute HCl. The blue solution becomes colourless without any yellow coloration.

Experiment 106.—Mix equal quantities of HCl and lactic acid, and repeat Experiments 103, 104, 105. Record the results.

Experiment 107. Separation of Hydrochloric and Lactic Acids.—If both acids are present, free HCl can still be recognised by Günzburg's test, but it is difficult to obtain satisfactory results with the tests for lactic acid. In order to test for lactic acid, it is preferable to separate the two acids. This can be done as follows: To 5 c.c. of the fluid add an equal amount of ether. Close the test tube with the finger and shake vigorously. The lactic acid passes into the ether. Allow the two fluids to separate, remove the supernatant ether by means of a pipette, and allow the ether to flow into a beaker containing a small amount of hot water. (All flames in the neighbourhood must be extinguished.) The ether evaporates, leaving a watery solution of lactic acid, to which the various tests are applied.

Experiment 108.—Apply Tests 103, 104, 105, 107 to gastric contents or to a gastric digest.

Products of Peptic Digestion.

2. Products of Peptic Digestion.—After the fibrin shreds in the test tube A (from Exp. 94) have dissolved completely, the contents are collected in a beaker. The reaction is acid: test with litmus. Examine this peptic digest as follows:—

Experiment 109. Albumoses and Peptones.—Neutralise

carefully with dilute NaOH. A precipitate of *acid albumin* may appear when the solution is just neutral. An excess of NaOH must be avoided, as it would dissolve the acid albumin. Remove the precipitate of acid albumin by filtration. Boil the filtrate to remove any undigested coagulable protein that may be present. Filter, and use the filtrate for the examination of the products of peptic digestion: *albumoses* and *peptones*.

Albumoses.

Apply *tests for proteins*—biuret, xanthoproteic, Millon's hydroferro-cyanic acid. Note that in the xanthoproteic test the yellow precipitate which is formed in the cold, dissolves on heating and reappears on cooling.

Precipitation by Alcohol.—Add to some of the digest an excess of alcohol. Allow the precipitate which forms to stand in contact with alcohol for some time. Decant alcohol; the precipitate dissolves in water. Compare with simple protein and state the difference.

Solubility in Ammonium Sulphate Solution.—Determine solubility in saturated ammonium sulphate solution, by saturating the digest with *solid* ammonium sulphate. A precipitate of albumoses appears.

Peptones.

If digestion has proceeded sufficiently far, the filtrate from this precipitate will still give the biuret test (an excess of strong alkali must be used in applying this test here. Why?) and the xanthoproteic test. Note that in the latter test the nitric acid does not give a yellow precipitate at all, but gives only a yellow solution.

The following scheme summarises the method of examining a peptic digest:—

METHOD OF EXAMINATION OF PEPTIC DIGEST.

Neutralise carefully. If a precipitate appears, *acid albumin* is present.

Filter, if necessary, and boil. If a precipitate appears, *unaltered coagulable protein* is present.

Filter, if necessary, and saturate with solid ammonium sulphate. If a precipitate appears, *albumoses* are present.

Filter, if necessary, and apply to filtrate biuret and xanthoproteic tests. If tests are positive, *peptones* are present.

Experiment 110.—Examine by means of this scheme the different peptic digests which are given out, and state how far digestion has proceeded in these digests.

Experiment 111. Dialysis.—(Demonstration.) Dialyse globulins, albumins, albumoses, and peptones in parchment tubes against water. Examine the dialysate for proteins by applying—1, biuret test; 2, ninhydrin test.

Dialyse a mixture of starch, glucose, and chlorides in the same way against water, and examine the dialysate.

Properties of Proteins.

From these experiments, and from Experiments 33 to 38, 85 and 86, summarise the properties of different proteins accordingto the following table:—

Variety of Protein.	Biuret Test.	HNO_3.		Solubility in $(NH_4)_2SO_4$.	Dialysis Indicating Colloidal Nature.	Alcohol.	Coagulation by Heat.
		Precip.	Heat.				
Globulins -							
Albumins -							
Albumoses							
Peptones -							

PANCREATIC DIGESTION.

PANCREATIC JUICE.

An alkaline extract of pancreas is supplied.

Determine experimentally the following points, and record your results. (For the arrangement of these experiments see experiments on saliva and gastric juice.)

Experiment 112. Does the Extract contain a Proteolytic Ferment (Trypsin)?—(Digestion of fibrin, see Exp. 94.)

Experiment 113.—If so, how is the activity of the ferment affected—(*a*) by boiling; (*b*) by cold; (*c*) by acting in a faintly acid medium?

Experiment 114.—Does the Extract contain an Amylolytic Ferment (Amylopsin)?—(Digestion of starch, see Exp. 89.)

Experiment 115.—If so, how is the activity of the ferment affected—(*a*) by boiling; (*b*) by cold; (*c*) by acting in a faintly acid medium?

Experiment 116. Does the Extract contain a Lipolytic Ferment (Steapsin)?—(For this experiment a fresh extract is necessary, as lipase is rapidly destroyed.) Proceed as follows:—

Boil 10 c.c. of fresh milk in order to destroy any lactic acid bacilli which may be present. Why is this necessary? Cool and add 2 c.c. of *neutral* extract of pancreas and a few drops of litmus solution. Owing to the alkalinity of the milk, the mixture should now be faintly alkaline, as indicated by the blue colour of the litmus.

Divide into two portions, and label one tube "Ferment," the other "Control." Heat the "Control" tube to boiling, then cool under the tap. Place both tubes in a water-bath at 37° to 40°. If a lipolytic ferment is present the blue

colour in the tube labelled "Ferment" changes to red, owing to the formation of fatty acids from the fat of the milk. The change in colour is much more evident by comparison with the "Control" tube in which the colour has remained unaltered since the ferment has been destroyed by boiling.

PRODUCTS OF TRYPTIC DIGESTION OF PROTEIN.

Experiment 117.—Prepare a pancreatic digest by allowing pancreatic extract to act on fibrin for two or three hours.

Examine the tryptic digest according to the scheme given in Experiment 109. Note, however, that digestion has proceeded in an alkaline medium, so that any precipitate which appears on neutralising with dilute acid must be alkali albumin, not acid albumin, as in the case of a peptic digest. Record your results.

METHOD OF TESTING FOR FERMENTS —CONTROL EXPERIMENTS.

Since ferments cannot be isolated as such, their presence is recognised by their action. In order to test for the presence of a ferment, the fluid to be tested is brought together in a test tube with the suitable substrate, for instance starch, if one wishes to test for an amylolytic ferment. A second experiment, a so-called "control experiment," is performed simultaneously in order to exclude possible fallacies. In the control experiment the same quantity of substrate is brought together with the same quantity of the fluid, in which, however, the ferment has been destroyed by previously boiling the fluid. The two tubes are allowed to remain at the same temperature

for the same period of time, and are then examined by suitable methods in order to see whether any change has taken place in the substrate. Since the conditions in the two tubes are exactly the same, except that the control tube is certainly free from ferments, any difference between the two tubes can be taken as evidence for the presence of a ferment in the fluid to be tested. An example is given in Experiment 116.

The substrate in the control tube may not show any change at all; in such a case a qualitative difference between the two tubes is sufficient evidence for the presence of a ferment. If, on the other hand, the substrate in the control experiment shows a change, which must then be due to substances other than ferments, a quantitative evaluation of the changes produced in the two experiments is necessary. The conclusion that a ferment is present can in such a case be drawn only if the change produced in the main experiment is greater than that produced in the control experiment.

BILE.

Ox or sheep bile is supplied.

Experiment 118.—Note the green colour. The colour is due to the pigments biliverdin and bilirubin. What is the relation between biliverdin and bilirubin?

Test the reaction with glazed litmus paper by allowing a drop of bile to lie on the paper for about a minute, and removing it with some water. Record. Note taste.

Acidify some bile with acetic acid. A precipitate of a mucinoid protein appears. It is insoluble in excess of the acid.

Experiment 119. Effect of Bile on Surface Tension.—Fill a test tube with water. Sprinkle some flowers of sulphur on the surface. The particles float.

Fill a test tube with alcohol and sprinkle some flowers of sulphur on the surface. The particles sink. This is due to the fact that alcohol has a lower surface-tension than water. What is surface-tension?

Fill a test tube with water to which some bile has been added. Sprinkle some flowers of sulphur on the surface. The particles sink. What effect has bile on the surface-tension of water?

Experiment 120.—Into two funnels place filter papers. Moisten the one with water, the other with diluted bile. Pour some oil into each. The oil passes through the filter paper moistened with bile, but not through that moistened with water. Why?

Experiment 121. Effect of Bile on Emulsification.—Shake up some olive oil containing some fatty acids—(*a*) with water, (*b*) with 1 per cent. sodium carbonate, (*c*) with some bile. In (*a*) no emulsion is formed, in (*b*) and (*c*) an emulsion is formed. Emulsification is more complete in (*c*) than in (*b*). Explain.

What bearing has this fact on the absorption of fat from the intestine?

Experiment 122. Bile Salts.—Pettenkofer's test. Dilute some bile with water, and in 5 c.c. of this diluted bile dissolve a fragment (less than half) of a crystal of cane sugar. Shake. When the cane sugar has dissolved incline the test tube and allow 5 c.c. of concentrated sulphuric acid to flow down the side of the tube, so that the acid settles to the bottom. Gently shake the test tube from side to side. A purple colour develops where the acid mixes with the bile, both in the fluid and in the froth which was formed as the result of shaking.

Note 1.—Care must be taken that in mixing the bile

with the sulphuric acid the temperature does not rise above 70°.

Note 2.—A solution of cane sugar alone, if mixed with concentrated sulphuric acid in the same way, gives a black ring at the junction. Carry out this test with cane sugar alone. In testing for bile salts it is therefore necessary to avoid an excess of cane sugar.

Why is cane sugar necessary for this test, and by what substance can it be replaced?

Note 3.—This test cannot well be applied to urine, since urine contains other substances which give a similar colour reaction. If applied to urine it is best carried out in the following modification, which should here be carried out with bile. Dissolve a small fragment of a cane sugar crystal in dilute bile, then filter repeatedly through a filter paper. Allow the filter paper to dry by placing it over the radiator. To the dried paper apply a drop of concentrated sulphuric acid. After a few seconds a violet colour appears, which can best be seen in transmitted light. (Normal urine gives a reddish or brownish colour.)

Experiment 123. Bile Pigments. Gmelin's Test.—Place in a test tube 5 c.c. of strong nitric acid containing some nitrous acid (*i.e.*, fuming yellow nitric acid). With a pipette carefully add 5 c.c. of dilute bile, so that the bile floats on the top of the nitric acid. Gently shake the test tube from side to side. At the junction of the two liquids a series of colours appears, spreading up into the bile. (At the same time a white ring of the precipitated mucin of the bile is seen. This, however, has nothing to do with the bile pigment.) The test depends upon the oxidation of bilirubin, which gives rise to biliverdin and other pigments. The play of colours is best seen with bile which has a brown colour. Why? In that case the colours seen from the bile to the acid are green, blue, red, and yellow.

The test can also be carried out as follows:—

A drop of dilute bile is spread out on a white porcelain slab in a thin film. A drop of fuming nitric acid is placed on it. A play of the various colours is seen round the drop of nitric acid.

Another modification of the same test is to filter the dilute bile repeatedly through a filter paper. Allow the filter paper to dry superficially, and then place a drop of fuming nitric acid on it. A similar play of colours is obtained.

Experiment 124. Huppert's Test for Bile Pigments.—Render some dilute bile alkaline with a little caustic soda or sodium carbonate; add an equal amount of barium chloride or calcium chloride solution. A precipitate forms which carries down the pigment. Filter, wash the precipitate in the filter paper with water. Transfer the precipitate to a test tube, and add to it about 5 c.c. of alcohol. Add five drops of concentrated HCl and shake. The precipitate dissolves. Add two drops of ferric chloride. Heat the alcoholic solution. The solution becomes green. This is the most reliable test for bile pigment.

Experiment 125. Cholesterin.—In a capsule evaporate 10 c.c. of bile to dryness on the water-bath. Cool, and extract the residue with ether. Decant the ethereal solution into a beaker and again extract with ether. Decant again, collect the ethereal extracts, and allow the ether to evaporate. (*All gas flames must be extinguished.*) When the ether has been evaporated a residue remains, which should be dissolved in a few drops of warm alcohol. Examine microscopically the crystals which separate out on cooling, and apply the tests for cholesterin.

Under certain pathological conditions cholesterin separates out from bile. What is the result?

NORMAL METABOLISM.

TISSUE RESPIRATION.

(Demonstration.)

OXYGEN ABSORPTION.

The avidity with which tissue cells absorb oxygen can be demonstrated by suspending them in a salt solution tinged with methylene blue. The tissue cells by absorbing oxygen reduce the coloured methylene blue to the colourless leuko-base. The experiment is carried out as follows :—

Prepare a Locke solution containing 0·001 per cent. methylene blue. A number of long, narrow test tubes (20 cm. long, internal lumen 0·5 cm.) are filled with this solution. About 0·3 to 0·5 gm. of a finely mixed emulsion of kidney cells, freed as much as possible from blood, is introduced into the tube and made to sink to the bottom of the tube. A few drops of liquid paraffin are added, forming a layer at the top of the tube, and thus excluding the access of air. The tube is kept at 37°. After fifteen to thirty minutes the blue colour disappears, at first immediately above the cells at the bottom of the tube. The decolorisation spreads slowly upwards.

Remove some of the decolorised fluid by means of a pipette, and transfer it to a test tube. On contact with the air the solution quickly becomes blue again.

This experiment can be used to demonstrate a number of phenomena.

Difference in the Rate of Oxygen Absorption by Different Tissue Cells.—Add approximately equal quantities to three test tubes containing the coloured Locke solution. The rate at which the colour disappears in the three tubes differs, indicating a difference in the rate of oxygen absorption. It is quickest with kidney cells, slower with brain and liver cells.

Respiration in Cold-Blooded and Warm-Blooded Animals.—Compare the effect of kidney cells from a warm-blooded animal and a cold-blooded animal (frog). With the latter the colour of the methylene blue disappears if the tube is kept at room temperature, but not with the former.

Action of Narcotics.—Shake the emulsion of kidney cells fifteen minutes with a Locke solution saturated with chloroform. Then add the chloroformed kidney cells to the methylene blue Locke solution. The oxygen absorption is now retarded, as indicated by the slower disappearance of the colour.

Action of Inorganic Salts. Antagonism between Ca and Na Ions.—Shake the emulsion of kidney cells for fifteen minutes in a 1·6 per cent. solution of $CaCl_2$. Remove one portion of the emulsion, place in the methylene blue Locke solution, and keep tube in the cold. The remainder of the kidney emulsion is shaken with 0·9 per cent. NcCl solution in tap water for fifteen minutes, and then placed in the methylene blue Locke solution. Incubate both tubes at 37°. The oxygen absorption of the cells treated with Ca ions is greatly retarded. The rate of respiration of the cells treated first with Ca ions and then with Nc ions has

been partially restored, the Na ions having antagonised the action of the Ca ions.

Respiratory Function of the Blood Pigment.—This can be demonstrated by the same experimental arrangement, using instead of the methylene blue Locke solution a Locke solution containing oxyhæmoglobin in such a dilution that the two bands are just distinctly visible. An emulsion of kidney cells is added to the solution, and the test tube incubated at 37° for fifteen minutes. If now the absorption spectrum of the fluid is projected on a screen, the upper part of the fluid will still show the absorption spectrum of oxyhæmoglobin, while immediately above the kidney cells the characteristic two bands of oxyhæmoglobin are replaced by the less distinct band of hæmoglobin.

CO_2 Excretion.—Place a weighed amount of an emulsion of kidney cells in a Gooch crucible. Add a few drops of Ringer's solution. It is necessary to use the original Ringer's solution, not any of the modifications containing carbonates or phosphates which would absorb the CO_2 formed. Pipette 30 c.c. of saturated baryta water into each of two large Erlenmeyer flasks (capacity, 1,000 c.c.) Suspend the Gooch crucible over the baryta water in one of the flasks by means of a small wire ring fixed to the stopper of the flask. The other flask serves as a control. Stopper both flasks tightly. Incubate both flasks at 37° for fifteen to thirty minutes. The baryta water in the control flask has remained clear, while that in the flask containing the kidney cells is turbid, as the result of the precipitation of barium carbonate. The amount of CO_2 excreted can be determined quantitatively by removing 10 c.c. from each flask and titrating with a standard oxalic acid solution, using methyl orange as indicator. The difference in the amounts of standard oxalic acid used indicates the amount

of baryta which has combined with the CO_2. The standard of the oxalic acid is such that 1 c.c. acid indicates 5 c.c. CO_2.

(Preparation of standard oxalic acid: Dissolve 28·19 gm. crystallised oxalic acid in 1,000 c.c. freshly boiled distilled water. Preparation of saturated baryta water: 6·3 gm. are shaken in 1,000 c.c. water and allowed to stand for several days. The supernatant fluid is pipetted off.)

URINE.

CONSTITUENTS OF NORMAL URINE.

Each student is expected to provide for these experiments about 200 c.c. of his own urine.

Colour.—The colour is of a transparent yellow. The froth which forms on shaking soon disappears. Normal urine contains no sediment when passed. Deposits may form later (see below).

Experiment 126. Reaction.—The urine is acid to litmus. Test. It may react acid to blue litmus, and alkaline to red litmus: "amphoteric reaction." During the day it becomes less acid, and may even have an alkaline reaction.

On standing urine becomes alkaline unless kept sterile. This is due to "ammoniacal fermentation," produced by the *Micrococcus ureæ*, which converts urea into ammonium carbonate.

Experiment 127. Specific Gravity. Total Solids.—Determine the specific gravity by means of a urinometer, as described in Experiment 84. The urinometers are graduated for a temperature of 15° C.

The specific gravity of normal urine varies between 1·015 and 1·025, and gives a rough indication of the amount of solids present in the urine. The last two

figures of the specific gravity multiplied by 2·33 give approximately the solids in grammes per litre.

Example: specific gravity 1·020.

20 : 2·33 = 46·6 gm. of solids per litre of urine.

Volume.—The volume varies. The average volume is 1,200 to 1,400 c.c. in twenty-four hours.

CHLORIDES.

Experiment 128.—To 15 c.c. of urine add a drop or two of concentrated nitric acid (if the urine is alkaline add nitric acid till reaction is acid) and five drops of silver nitrate solution (2 per cent.). A coherent clump of silver chloride is precipitated if the chlorides are present in normal quantity. If the chlorides are much diminished, as in febrile conditions, the precipitate is more or less diffuse, according to the diminution. Why is nitric acid added? Add ammonia; the precipitate dissolves.

What is the average quantity of chlorides in normal urine? What is the source of the chlorides in urine?

SULPHATES.

Experiment 129. (*a*) **Inorganic Sulphates.**—To 10 c.c. of urine add about 3 c.c. of barium chloride solution. A thick precipitate forms, which consists of the phosphate and sulphate of barium. Acidify with a few drops of concentrated HCl. The barium phosphate dissolves and an opaque milkiness remains, indicating the presence of inorganic sulphates. If the precipitate which remains is thick the inorganic sulphates are in excess.

Experiment 130. (*b*) **Ethereal Sulphates.**—To 10 c.c. of urine add barium chloride as long as a precipitate continues to form. Make alkaline with a few drops of sodium carbonate solution, and filter. Acidify the filtrate,

which is now free from inorganic sulphates, with concentrated HCl, and boil for three minutes. A faint cloud of barium sulphate is formed on standing, indicating the presence of ethereal sulphates. If ethereal sulphates are present in excess, a distinct precipitate is formed, which settles to the bottom.

What is the average daily quantity of sulphates in urine? What is the source of the sulphates in urine? What does an excess of ethereal sulphates indicate?

Experiment 131.—Prepare an ethereal sulphate as follows:—

Warm ten drops of absolute alcohol with five drops of concentrated sulphuric acid. After cooling, make alkaline with 10 per cent. sodium hydrate; add barium chloride as long as a precipitate continues to be formed; then heat to boiling point, and filter. The filtrate contains barium ethyl-sulphate. To it add half its volume of concentrated hydrochloric acid, and boil. A precipitate of barium sulphate forms. Compare the solubility in water of barium sulphate and barium ethyl-sulphate.

PHOSPHATES.

Experiment 132.—To a test tube full of urine add a little strong ammonia and heat. A white precipitate of the phosphates of calcium and magnesium—"earthy phosphates"—forms. (Such a precipitate is often found in alkaline urine as a crystalline deposit. See below, under Deposits.) Filter. The filtrate contains the phosphates of sodium and potassium—"alkaline phosphates."

A. *Precipitate.* "*Earthy Phosphates.*"—Place the precipitate in a test tube with some water, and add a few drops of dilute acetic acid. It dissolves. The presence of phosphoric acid in this solution can be verified by the

ordinary tests; for instance, by the formation of ammonium phosphomolybdate. To the acid solution add some nitric acid and about 5 c.c. of ammonium molybdate. Heat gently over a small flame to about 60°. The solution turns yellow, and a yellow crystalline precipitate is formed. What is the yellow precipitate?

B. *Filtrate.* "*Alkaline Phosphates.*"—To the filtrate add a little magnesia mixture and warm gently. A white precipitate is formed, indicating the presence of phosphates in the filtrate. What is this precipitate? The presence of phosphates may also be determined by testing with ammonium molybdate as detailed above.

Note the difference in the bulk of this precipitate and that of "earthy phosphates" obtained on adding ammonia to the urine. Are the "earthy" or the "alkaline phosphates" present in larger amount in urine?

Experiment 133. Formation of Deposit of Ammonium Magnesium Phosphate. ("**Triple Phosphate**").—Set some urine aside in a beaker for two or three days. Ammoniacal fermentation occurs (the reaction of the urine becomes alkaline; test with litmus), and a crystalline deposit of ammonium-magnesium phosphate settles out. Examine microscopically. Sketch. Crystals of calcium phosphate may also be present, and may be recognised by the stellar arrangement of the crystals.

Experiment 134.—On heating neutral urine a precipitate of earthy phosphates is often formed. Try this. It dissolves on the addition of a few drops of acetic acid. This is important in testing for albumin by the heat test.

The explanation for the appearance of this precipitate is given by the following experiment:—

Experiment 135.—Treat a solution of calcium chloride with sodium phosphate, and then with excess of sodium

carbonate. Calcium phosphate is precipitated. Add acetic acid, drop by drop, till the precipitate just dissolves: acid calcium phosphate is formed. Heat: calcium phosphate is precipitated again owing to the alteration of the reaction as the carbon dioxide is evolved. The precipitate dissolves on adding a drop or two of dilute acid.

Experiment 136. — Precipitation of Phosphates by Uranium Nitrate.—To a little urine add a few drops of acetic acid and some sodium acetate, and then uranium nitrate. A precipitate forms. In order to complete precipitation the urine must be heated to boiling point.

This reaction is used for the quantitative estimation of phosphates.

What is the average daily quantity of phosphates in the urine?

What is the source of phosphoric acid in the urine?

By which other channels are calcium and magnesium excreted?

DEPOSITS OF PHOSPHATES IN URINE.

Urinary Deposits of Phosphates.

From alkaline urine deposits of phosphates may separate out. All these deposits are easily soluble in acetic acid.

Earthy Phosphates.—Amorphous granules of $Ca_3(PO_4)_2$ and $Mg_3(PO_4)_2$.

Ammonium-Magnesium Phosphate, $MgNH_4PO_4$. (Triple phosphate.)—Large colourless prisms in the shape of "knife-rests" or "coffin lids," or in the shape of feathery crystals.

Calcium-Hydrogen Phosphate, $CaHPO_4$. — Prismatic crystals arranged in rosettes. These may also occur in acid urines.

DEPOSIT OF OTHER CALCIUM SALTS IN URINE.

Urinary Deposits of Calcium Salts.

From *acid* urines:—

Calcium oxalate, either in the form of highly refractive octahedra, "envelope" shape, or in the form of ovoid bodies, "dumb-bell" shape. They are insoluble in acetic acid; soluble in hydrochloric acid. A considerable sediment of calcium oxalate is pathological.

From *alkaline* urine:—

Calcium carbonate.—Spherical or ovoid crystals with concentric striation. Readily soluble in acetic acid with effervescence. Common deposit in the urine of herbivorous animals.

AMMONIA.

Experiment 137.—Make urine alkaline with sodium carbonate solution, and warm. Note the smell of ammonia. A moist piece of red litmus paper held over the mouth of the tube turns blue.

Within what limits does the average amount of ammonia excreted by a normal person in twenty-four hours vary? How can it be increased? How can it be diminished?

PREPARATION OF UREA FROM URINE.

Experiment 138.—Place about 100 c.c. of urine in a porcelain capsule and evaporate in a water-bath to about 20 c.c., so that the urine is now concentrated to a syrup. Cool by floating the capsule on cold water. Filter into a beaker, and place beaker in cold water. Slowly add while stirring an equal volume of cold 50 per cent. nitric acid (pure). (Why can fuming yellow nitric acid not be used?)

The mixture must be kept cold. Crystals of urea nitrate separate out. Filter off the crystals; dry them by pressing between successive sheets of filter paper. When the crystals are dry, mix with excess of barium carbonate, and add a little alcohol to form a paste. The urea nitrate is decomposed, giving barium nitrate, CO_2, and urea. Dry the mass on the water-bath, extract with alcohol, and filter. Concentrate on the water-bath the alcoholic filtrate to a small volume. Cool. Urea crystallises out in long needles. Examine microscopically. Sketch the crystals. If no immediate crystallisation occurs, allow the alcoholic solution to stand overnight.

Is urea or urea-nitrate more soluble in water?

Within what limits does the amount of urea excreted by a normal person in twenty-four hours vary? How can it be increased? How can it be diminished?

PREPARATION OF URIC ACID FROM URINE.

Experiment 139.—To 25 c.c. of urine in a beaker add 5 c.c. of strong HCl, and allow to stand for twenty-four hours. Examine microscopically the crystals of uric acid which separate out. Sketch the crystals. Note that the crystals are deeply pigmented with urinary pigment.

To some of the crystals add caustic soda. The crystals dissolve. To the alkaline solution add excess of strong HCl. The crystals are again formed after some time.

The crystals give the murexide test (see below, Exp. 146).

PREPARATION OF AMMONIUM URATE FROM URINE.

Experiment 140.—To 25 c.c. of urine add two drops of ammonia and solid ammonium chloride, stirring all the

time until the solution is saturated. Avoid an excess of the salt. A gelatinous, amorphous precipitate of ammonium urate is formed. Examine microscopically. All the uric acid present in the urine is completely precipitated as ammonium urate. This reaction is therefore used for the quantitative estimation of uric acid.

The deposit gives the murexide test (see below).

What is the source of the uric acid excreted in urine?

Within what limits does the quantity of uric acid excreted in twenty-four hours by a normal person vary? How can it be increased? How can it be diminished?

UREA.

What is the structural formula for urea?

Prepare biuret from urea (see Exp. 34, *a*).

Experiment 141. Solubility.—Determine the solubility of urea in (*a*) water; (*b*) cold alcohol; (*c*) cold ether. Record the results.

Prepare a dilute watery solution of urea for the following experiments.

Experiment 142.—To 5 c.c. of the urea solution add some sodium hypobromite. Bubbles of nitrogen are evolved.

State in the form of an equation the process which takes place. This reaction is used for the quantitative estimation of urea. It is, however, given, not only by urea, but by all substances having amido groups and by ammonium salts.

Repeat the test, using a dilute solution of ammonium sulphate instead of the urea solution. Record the result.

Experiment 143.—To some urea solution add some yellow nitric acid, which contains nitrous acid. Bubbles of nitrogen and of CO_2 are evolved.

This reaction is also given by all substances having an amido group.

State in the form of an equation the process which takes place.

Experiment 144.—Boil some urea solution with caustic soda. Notice the smell of ammonia which is evolved.

State the reaction which takes place.

URIC ACID.

What is the structural formula for uric acid?

Experiment 145. Solubility.—Determine the solubility of uric acid in (*a*) water, cold and hot; (*b*) alcohol, cold and hot; (*c*) caustic soda or sodium carbonate, cold and hot; (*d*) dilute HCl, cold and hot.

Dissolve some uric acid in warm caustic soda, acidify with dilute HCl, and allow to cool slowly. Uric acid crystallises out. Examine microscopically. Sketch the crystals.

What is formed when uric acid is dissolved in caustic soda?

Experiment 146. Murexide Test.—To a small crystal of uric acid on a chip of thin porcelain or a crucible lid, add two drops of strong nitric acid. Evaporate over a *small* flame, in a fume chamber, to complete dryness. A red deposit remains. Add with a glass rod a drop of *very dilute* ammonia. The residue turns to a violet colour.

Experiment 147. Uric Acid reduces Fehling's Solution.—Dilute some Fehling's solution and heat to boiling point. To the boiling solution add repeatedly a few drops of a solution of uric acid in caustic soda, heating after each addition. Red cuprous oxide separates out. If the urate is present in excess a white precipitate of cuprous urate will be formed at the same time.

The presence of uric acid may therefore be a fallacy in examining urines for sugar by means of Fehling's test,

especially if the boiling is prolonged. This can be shown to be the case as follows :—

Experiment 148.—Heat simultaneously in two test tubes equal volumes of normal urine and of half-diluted Fehling's solution to boiling point. When boiling mix the contents. Pour one-half of this mixture of urine and Fehling's solution into a test tube and allow to cool. This will remain blue if the urine is normal. Boil the other half for about three minutes: if sufficient urates are present the solution may become decolorised and acquire a brown colour, owing to the reduction of the cupric salt by urates.

Experiment 149.—Boil 5 c.c. solution of uric acid in caustic soda with 1 c.c. of Nylander's reagent. No reduction takes place.

Experiment 150.—The presence of uric acid is therefore no fallacy if Nylander's reagent is used. Demonstrate this by adding to some urine (5 c.c.) one-tenth of its volume ($\frac{1}{2}$ c.c. = 10 drops) of Nylander's reagent; boil over a small flame for three minutes. No reduction to metallic bismuth occurs.

DEPOSITS OF URIC ACID AND URATES.

From *acid* urines a crystalline deposit of uric acid may separate out. It has a sandy red colour, and is therefore called "cayenne pepper deposit." This deposit corresponds to the one obtained experimentally in Experiment 139. It is recognised by its crystalline form and by the murexide test.

From *acid* urines a deposit of urates (mainly sodium urates) may separate out. It may be amorphous or crystalline. It has a pinkish-red colour, and is therefore called "brick dust deposit." This deposit is found frequently in concentrated urines on cooling. It dissolves

on heating the urine, and thus differs from deposits of phosphates.

The deposit is soluble in hot water or hot acids. On adding hydrochloric acid to the watery solution, and allowing to cool, crystals of uric acid separate out. The deposit gives the murexide test. It is not readily soluble in cold acetic acid.

From *alkaline* urines a deposit of ammonium urate may separate out in the form of yellow or brownish spheres, with or without projecting spicules—"hedgehog crystals." They dissolve in hydrochloric acid; on standing uric acid crystallises out of the acid solution. The deposit gives the murexide test.

A considerable sediment of uric acid or urates does not necessarily indicate a high uric acid content.

CREATININE.

What is its structural formula?

Experiment 151. Weyl's Test.—To 5 c.c. of urine add a few drops of a freshly prepared solution of sodium nitroprusside. Then render alkaline with caustic soda. A red colour results. Strongly acidify with acetic acid. The solution is decolorised.

Experiment 152. Jaffe's Test.—To 5 c.c. of urine add picric acid. Then render alkaline with caustic soda. The solution becomes red in colour.

What is the average amount of creatinine excreted by a normal person in twenty-four hours?

Creatinine, like uric acid, is capable of slightly reducing Fehling's solution. It has at the same time the power to keep in solution a small amount of cuprous oxide. If, therefore, a dilute glucose solution containing creatinine is tested with Fehling's solution, a brown clear solution

instead of a red or yellow deposit may be obtained. This may also occur if a urine of normal concentration containing only a small amount of sugar is examined by Fehling's test.

INDICAN.

Experiment 153.—To about 10 c.c. of urine add an equal volume of strong *fuming* HCl, one or two drops (not more!) of a 5 per cent. solution of calcium hypochlorite, and 3 c.c. chloroform. Close the mouth of the tube with the thumb, cautiously invert a few times, and allow the mixture to stand for a few minutes. The chloroform becomes blue. Note the change in the colour of the urine.

The amount of indican present is proportional to the depth of colour of the chloroform extract. A rough estimate of the amount of indican present can be obtained by comparing the depth of the blue colour with a blue standard solution, *e.g.*, Fehling's solution.

From what substance is the indican formed?

What relation does the amount of indican present in the urine bear to intestinal putrefaction?

Repeat the test, using about 5 c.c. of ferric chloride solution, instead of the calcium hypochlorite. The same result is obtained.

Repeat the test, with one or two drops of calcium hypochlorite, as detailed above. After the chloroform has become blue add a few more drops of calcium hypochlorite, and invert the tube repeatedly. What takes place?

Why is the addition of calcium hypochlorite necessary for this reaction, and by what substances can it be replaced?

Why must an excess of calcium hypochlorite be avoided?

Is the addition of chloroform necessary for the formation of indican?

QUANTITATIVE ESTIMATION OF CERTAIN NITROGENOUS CONSTITUENTS OF THE URINE.

Quantitative Urine Analysis.

Each student is expected to collect accurately a twenty-four hour specimen of his own urine, and to measure and record its volume.

The urine should be protected against ammoniacal fermentation by the addition of 2 c.c. of a 5 per cent. solution of thymol in chloroform, which is supplied. A sample (about 150 c.c.) of the total twenty-four hours' urine should be kept on hand for the experiments.

Decinormal solutions of acid and alkali are supplied.

NORMAL SOLUTIONS.

A normal solution is one which contains the equivalent weight in grammes of a given substance dissolved in 1,000 c.c. of water.

What is meant by the term "equivalent weight"?

As an example take hydrochloric acid. The equivalent weight of Cl = 35·5, H = 1, therefore HCl = 36·5. Therefore a normal HCl solution contains 36·5 gm. HCl in 1,000 c.c. of water, a decinormal solution contains 3·65 gm. HCl in 1,000 c.c. of water, and 1 c.c. of a decinormal HCl solution contains 3·65 mg. HCl.

Normal solution is written $n/1$, decinormal $n/10$, and so on.

The same holds good for, say, NaOH, or for H_2SO_4. Note that for a dibasic acid, such as H_2SO_4, the equivalent weight is half the molecular weight. What is the equivalent weights for NaOH? for H_2SO_4? for NH_3?

Since normal solutions contain equivalent weights dissolved in equal volumes, it follows that each c.c. of any

$n/10$ acid will require for neutralisation exactly 1 c.c. of any $n/10$ base. Try this as follows :—

Experiment 154.—Run from a burette into a beaker exactly 10 c.c. of $n/10$ acid. Dilute with about 20 c.c. of distilled water, add two drops of an indicator (phenolphthalein). Then add slowly from a burette $n/10$ alkali, until the addition of one drop will just produce a purple colour.

Record your result.

Experiment 155.—Repeat the titration, using rosolic acid as an indicator instead of phenolphthalein.

Total Nitrogen Estimation.

Experiment 156. Estimation of the Total Nitrogen by Kjeldahl's Method.

Principle. First Stage. Acid Incineration.—By boiling with concentrated sulphuric acid all organic nitrogenous compounds are converted into ammonium sulphate. This reaction is accelerated by the addition of a small amount of copper sulphate, which acts as a catalyst. (What is a catalyst?)

Second Stage. Distillation.—The ammonium sulphate formed in the first stage of the process is decomposed by the addition of an excess of caustic soda. The ammonia, which is set free, is distilled into a measured amount of standard $n/10$ sulphuric or hydrochloric acid. The amount of the acid that has been neutralised by the ammonia is found by subsequent titration with standard $n/10$ sodium hydrate.

Process. First Stage.—From the twenty-four hours' urine take exactly 5 c.c. by means of a pipette and place in a Kjeldahl flask in such a way that the urine does not

touch the sides of the neck of the flask. Add about 10 c.c. concentrated sulphuric acid (*measured with a measuring cylinder, not with a pipette*), and a small crystal of copper sulphate. Place the Kjeldahl flask in the fume-chamber and heat. After all the water is driven off a more or less violent reaction will take place. Continue the boiling until a clear, almost colourless, solution is obtained (30 to 45 minutes in the case of urine).

In the meantime arrange the apparatus for distillation by connecting the upper end of a condenser with a spurting bulb, while the lower end is connected with an "adapter," a glass tube, which passes down into a receiving flask.

Second Stage. Distillation.—Allow the incinerated urine to cool. In the meantime place in receiving flask from a burette an accurately measured quantity (between 25 c.c. and 50 c.c.) of $n/10$ acid, *e.g.*, 30 c.c. Note the amount of acid placed in the receiving flask. When the Kjeldahl flask has cooled add distilled water to the contents, so that about a third of the flask is filled. (The first addition of water must be done carefully, because the Kjeldahl flask contains concentrated sulphuric acid.) Add two drops of an indicator, *e.g.*, rosolic acid (which gives a red colour when alkaline), to the contents both of the Kjeldahl flask and of the receiving flask. Now add a teaspoonful of talc to the contents of the Kjeldahl flask (in order to prevent bumping), then make the contents strongly alkaline by the addition of strong caustic soda, and at once close the Kjeldahl flask by inserting the spurting bulb. About 40 c.c. of 45 per cent. caustic soda solution will be necessary, if 10 c.c. of conc. sulphuric acid have been used for incineration. (*Note.*—The addition of soda is best carried out through a funnel, so that the mouth of the flask remains dry, otherwise it will be difficult to keep the spurting bulb in position.) See that the tube

connected with the lower end of the condenser dips into the acid.

Now heat, at first carefully, then with a full flame. Continue boiling until all the ammonia is distilled over. This will last from 30 to 45 minutes. The distillation is complete when a piece of red litmus held against the mouth of the adapter no longer turns blue.

Note 1.—In distilling great care must be taken to prevent a "sucking back" of the contents of the adapter into the distilling flask. This will occur as soon as the pressure in the distilling flask is allowed to fall by removing the flame or by careless heating. If there is any danger of "sucking back," the receiving flask should be lowered, so that the adapter does not dip into the acid.

Note 2.—If during distillation the contents of the receiving flask turn pink, indicating that the reaction has become alkaline, a measured amount of standard acid must be added at once. Otherwise ammonia will be lost.

When distillation is complete, remove first the receiving flask, then turn out the gas. Titrate the acidity of the contents of the receiving flask, and from that calculate the total amount of nitrogen excreted in twenty-four hours.

Calculation. Example :—

Total volume of twenty-four hours' urine = 1,550 c.c.
Amount taken for estimation = 5 c.c.
Amount of $n/10$ acid placed in receiving flask = 30 c.c.
Amount of $n/10$ alkali used in titration = 17·8 c.c.

Therefore amount of $n/10$ acid neutralised by ammonia = 12·2 c.c.

Now 1 c.c. $n/10$ acid = 1 c.c. $n/10$ ammonia.

Since 1,000 c.c. $n/10$ ammonia contain 1·7 gm. NH_3, 1 c.c. $n/10$ ammonia contains 1·7 mg. NH_3.

Therefore 1 c.c. $n/10$ acid = 1·7 mg. NH_3.

Further, 1·7 mg. NH_3 contains 1·4 mg. N, because the atomic weights are N = 14, H_3 = 3.

Therefore 1 *c.c. n/10 acid indicates* 1·4 *mg. N.*

Therefore the amount of N in milligrammes is obtained if the number of c.c. of n/10 acid, neutralised by ammonia, is multiplied by 1·4.

In this case 5 c.c. of urine contain 12·2 × 1·4 mg. N = 17·08 mg. N.

From this the total amount of nitrogen excreted in twenty-four hours can be calculated (in this case = 5·29 gm.).

Experiment 157. Estimation of Acidity and of Ammonia.—This method is based on the fact that ammonium salts (and also other substances containing an amido group) react with formaldehyde in such a way that the ammonia and formaldehyde form a complex organic compound (hexamethylentetramin or urotropin), while the acid which was combined with the ammonia is liberated. The amount of acid set free is determined by titration, and is a measure of the ammonia present.

In carrying out this estimation, the urine is at first neutralised by adding from a burette *n*/10 alkali. The amount of *n*/10 alkali added is a measure of the acidity of the urine. Then neutral formaldehyde is added. Owing to the liberation of acid which takes place when the formaldehyde has combined with the ammonia, the urine acquires again an acid reaction. This second acidity is titrated again with *n*/10 alkali, and this second titration is a measure of the amount of ammonia present. At least two estimations should be carried out.

Process.—Dilute 25 c.c. of urine with an equal volume of water, add 15 gm. of finely powdered neutral potassium oxalate in order to precipitate all the calcium salts, and four or five drops of phenolphthalein. Shake thoroughly

for one or two minutes, and, whilst the solution is still cold from the effect of the oxalate, titrate with $n/10$ NaOH until a permanent pink tint remains. Record the number of cubic centimetres added. This is a measure of the acidity.

Now dilute 10 c.c. of formalin with two volumes of water. This mixture will be slightly acid owing to the presence of some formic acid in the formaldehyde. It must be made neutral to phenolphthalein by adding $n/10$ NaOH until a faint permanent pink colour appears. Add this neutral formaldehyde to the urine. The urine becomes acid again and the colour disappears. Run the $n/10$ NaOH into the mixture until a permanent pink tint is again obtained. Record the number of cubic centimetres added in this second titration. This is a measure of the ammonia.

Calculate from your results :—

1. The titration *acidity* of the urine expressed in terms of $n/10$ acid (*a*) as percentage ; (*b*) for the total twenty-four hours' quantity.

2. The *ammonia* in grammes excreted in twenty-four hours.

3. The fraction of total nitrogen which is excreted as ammonia. Express the fractions in terms of percentage of total nitrogen.

Calculation. Example :—

Total volume of urine = 1,550 c.c.
Total N excreted in twenty-four hours = 5·29 gm.
Amount taken for estimation = 25 c.c.
First titration, 2·2 c.c. $n/10$ alkali.
Second titration, 7·7 c.c. $n/10$ alkali.

1. *Titration Acidity.*—Since 2·2 $n/10$ alkali neutralises 25 c.c. of urine, its titration acidity is 2·2 c.c. $n/10$ acid.

The acidity of 100 *c.c. of urine is therefore* 8·8 *c.c.* $n/10$ *acid.*

The acidity of the total urine is $\frac{8{\cdot}8 \times 1{,}550}{100}$ *c.c.* = 136·5 *c.c.* $n/10$ *acid.*

2. *Total* NH_3.—Since 1 c.c. $n/10$ alkali = 1 c.c. $n/10$ NH_3, 25 c.c. urine contains 7·7 c.c. $n/10$ NH_3.

1 c.c. $n/10$ NH_3 = 1·7 mg. NH_3.

Therefore 7·7 c.c. $n/10$ NH_3 = 7·7 × 1·7 mg. NH^3 = 13·09 mg. NH_3.

This is the amount present in 25 c.c. urine.

The amount excreted in twenty-four hours is therefore—

$$\frac{1{,}550 \times 13{\cdot}09}{25} \text{ mg. } NH_3 = 0{\cdot}811 \text{ gm. } NH_3.$$

3. *Ammonia N.*—Now 17 gm. NH_3 contain 14 gm. N.

Therefore 0·811 gm. NH_3 contain $\frac{14 \times 0{\cdot}811}{17}$ gm. N = 0·668 gm. N.

Of 5·29 gm. of total N, 0·668 gm. N are excreted in form of NH_3.

Of 100 gm. of total N, $\frac{0{\cdot}668 \times 100}{5{\cdot}29}$ gm. are excreted in form of NH_3 = 12·6 gm. N.

12·6 *per cent. of total N is excreted in the form of ammonia.*

Experiment 158. Estimation of Urea.—In the following method urea is estimated by measuring the amount of nitrogen liberated from the urine by sodium hypobromite. From 1 gm. urea 354 c.c. of nitrogen are evolved. As stated in Experiment 142 some other substances also liberate nitrogen if acted upon by hypobromite, so that the method gives only approximate results.

Apparatus.—Connect a bottle containing a short test tube, and closed with a rubber stopper, with an inverted burette standing in a tall glass cylinder filled with water. The apparatus is the same as the one used for the estimation of the oxygen capacity of the blood (Exp. 72).

The bottle is standing in a bath filled with water. The water in the jar and in the bath should have room temperature.

Method.—Place 20 c.c. of hypobromite solution in the bottle, without letting it touch the mouth of the flask. Run, with a pipette, 5 c.c. of urine accurately measured into a small test tube, and place the test tube into the bottle, taking care not to upset any of the urine into the hypobromite. Put in stopper tightly, place the bottle in the water-bath, connect one tube with the burette, leaving the second tube open. After five minutes close the second tube with a clip. Read the burette with water-level outside and inside equal.

Take the bottle out of the water-bath, and tilt, so that the urine mixes with the hypobromite. Gently shake bottle from side to side, holding it upright so that the froth does not enter the tube. Tilt again and shake again. Place the bottle back in water-bath. After five minutes read the burette again with water-level outside and inside equal. The difference in the readings gives the amount of N evolved. Record your results and calculate from them: (1) Amount of urea excreted in twenty-four hours; (2) that fraction of the total N which is excreted as urea.

Calculation. Example :—

Amount of urine used, 5 c.c.; N liberated, 8·7 c.c.
Volume of urine excreted in twenty-four hours, 1,550 c.c.
Amount of N excreted in twenty-four hours, 5·29 gm.

1. *Total Urea.*—Since 354 c.c. N are liberated by 1 gm. urea, 8·7 c.c. N are liberated by $\frac{8 \cdot 7}{354}$ gm. urea = 0·0245 gm. urea.

Since 5 c.c. of urine contain 0·0245 gm. urea, 1,550 c.c. urine contain $\frac{0 \cdot 0245 \times 1{,}550}{5}$ gm. urea = 7·61 gm. urea.

2. *Urea N in Percentage of Total N.*—Urea (molecular weight = 60) contains N_2 (molecular weight = 28).

Since, therefore, 60 gm. urea contain 28 gm. nitrogen, 7·61 gm. urea contain $\frac{7 \cdot 61 \times 28}{60}$ gm. N = 3·55 gm. nitrogen.

This amount of N excreted in twenty-four hours in the form of urea represents a fraction of the total N excreted in twenty-four hours. This fraction, expressed in terms of percentage of total N, is $\frac{3 \cdot 55 \times 100}{5 \cdot 29} = 67 \cdot 10$ per cent.

Result.—*The amount of urea excreted in twenty-four hours is* 7·61 *gm.*

The urea nitrogen represents 67·10 *per cent. of the total nitrogen.*

Collect the results from Experiments 156 to 158 in tabular form as under :—

NITROGEN DISTRIBUTION IN TWENTY-FOUR HOURS' URINE.

Date.	Volume.	Total N.	Urea.		Ammonia.	
			Absolute Amount.	N. Per Cent.	Absolute Amount.	N. Per Cent.

ON THE PRACTICAL SIGNIFICANCE OF THE NITROGEN DISTRIBUTION IN THE URINE.

The quantitative estimation of one single nitrogenous constituent—urea or ammonia—of the urine, which, in the case of urea, has long been the routine in clinical work, is of little practical use. In the case of both these substances, more especially in the case of urea, the absolute amounts excreted rise and fall with a rise and fall of the nitrogen intake, so that no definite conclusions can be drawn from a quantitative estimation of urea alone or ammonia alone. In order to be able to draw conclusions it is necessary to determine also the amount of total nitrogen excreted, and to calculate therefrom the fraction excreted in the form of urea and of ammonia respectively. If with a fairly constant excretion of total nitrogen the amount excreted in the form of urea diminishes, while that excreted in the form of ammonia increases, a severe

disturbance of metabolism is indicated, namely, a condition of *acidosis*.

If an estimation of total nitrogen is impracticable, estimation of urea and ammonia may give useful information. Normally the absolute amounts excreted rise and fall together, the rise being always more marked in the case of urea than in the case of ammonia. A fall in the urea excretion, accompanied by a rise in the ammonia excretion, indicates an acidosis.

It must be remembered that a high percentage of ammonia nitrogen—a so-called high "ammonia coefficient"—is not in itself evidence of a true acidosis. Such high ammonia coefficients always occur when the protein intake is greatly reduced, *i.e.*, with a low total nitrogen excretion.

QUANTITATIVE ESTIMATION OF CHLORIDES IN URINE.

Experiment 159. Estimation of Chlorides (Volhard's Method).—The principle of this method consists in precipitating all the chlorides with an excess of a standard silver nitrate solution. The excess of silver nitrate used is determined by adding a standard solution of ammonium sulphocyanate in the presence of a ferric salt. The sulphocyanate solution precipitates the soluble silver nitrate as silver sulphocyanate. As soon as all the silver nitrate is precipitated the addition of another drop of sulphocyanate solution will produce a red colour, since the ammonium sulphocyanate will now react with the ferric salt and form red ferric sulphocyanate.

The standard sulphocyanate solution is made up in such a way that 1 c.c. of it will just completely precipitate 1 c.c. of the standard silver nitrate solution (13 gm. NH_4CNS in 1,000 c.c.).

The standard silver solution is made up in such a way that 1 c.c. will completely precipitate 10 mg. NaCl (29·04 gm. $AgNO_3$ in 1,000 c.c.).

Method.—With a pipette place 10 c.c. of urine in a beaker, dilute with about 90 c.c. of distilled water, add 5 c.c. of a five per cent. solution of ammonia iron alum and 5 c.c. of dilute pure nitric acid. Then add with a pipette a measured excess of standard silver nitrate solution (20 c.c. of standard $AgNO_3$ will, as a rule, be sufficient). Now run in at once from a burette the standard sulphocyanate solution, stirring all the time, until the reddish tint of the ferric sulphocyanate first extends through the whole liquid. This can be observed most readily by comparing the urine to be titrated with another urine in which the end point has not yet been reached. Note the number of c.c. of sulphocyanate solution used, and calculate in terms of sodium chloride (1) the amount of chlorides in 100 c.c. urine; (2) the amount of chlorides excreted in twenty-four hours.

Calculation.—The amount of ammonium sulphocyanate solution used gives at once the excess of silver nitrate used beyond the quantity required to precipitate all the chlorides in 10 c.c. urine. If this excess is deducted from the total amount of silver nitrate added, one obtains the number of c.c. of standard silver nitrate solution necessary to precipitate all the chlorides.

Each c.c. of the standard silver solution equals 10 mg. NaCl.

Example :—

Urine used, 10 c.c.; total volume, 1,550.
Standard $AgNO_3$ added, 20 c.c.
Sulphocyanate solution used, 7·4 c.c.

Therefore excess of $AgNO_3$ solution = 7·4 c.c.

$AgNO_3$ solution necessary to precipitate all chlorides = 20 c.c. – 7·4 c.c. = 12·6 c.c.

Since 1 c.c. $AgNO_3$ = 10 mg. NaCl,
12·6 c.c. $AgNO_3$ = 126 mg. NaCl.

Since 10 c.c. of urine contain 0·126 gm. NaCl,
100 c.c. of urine contain 1·26 gm. NaCl.

Result.—*The total amount of chlorides excreted in twenty-four hours is in terms of NaCl* :—

$$\frac{1{\cdot}26 \times 1{,}550}{100} = 9{\cdot}53 \text{ gm.}$$

PATHOLOGICAL METABOLISM.

GASTRIC CONTENTS.

ESTIMATION OF HYDROCHLORIC ACID SECRETION.

In certain pathological conditions the gastric cells secrete more hydrochloric acid than they do normally, while in certain other conditions the amount of hydrochloric acid secreted by these cells is below the normal. The existence of such abnormal conditions can be recognised by a quantitative chemical examination of the gastric contents collected, by means of the stomach tube, an hour after a test meal of a fixed composition (for instance, dry toast and a large cup of tea without milk or sugar) has been given.

The chemical examination consists in estimating quantitatively :—

1. The total amount of hydrochloric acid secreted.

   Hydrochloric acid may exist in the gastric contents in three forms—(*a*) free HCl ; (*b*) HCl combined with proteins and organic bases ; (*c*) HCl combined with inorganic bases, as NaCl.

2. The "total acidity," which is a measure of the

"physiologically active HCl," provided that no other acids are present.

The physiologically active HCl comprises free HCl and HCl combined with proteins, etc.

3. The "free acidity," which is a measure of the "free HCl."

Why are butter, milk, and sugar excluded from the test?

Experiment 160. Estimation of Total Acidity. "Physiologically Active HCl."—Place into a beaker by means of a pipette exactly 10 c.c. of the filtered gastric contents. Add two drops of phenolphthalein. Titrate with $n/10$ sodium hydrate until a purple colour is just produced. Take the burette reading.

Record your result.

Calculation.—From your result calculate the number of c.c. of $n/10$ alkali necessary to neutralise 100 c.c. of gastric contents. From this calculate the weight in grammes of HCl which the total acidity represents.

Example.—6·5 c.c. $n/10$ alkali neutralise 10 c.c. gastric contents.

Therefore 100 c.c. gastric contents are neutralised by $6{\cdot}5 \times 10 = 65$ c.c. $n/10$ alkali.

Now 1 c.c. $n/10$ alkali = 1 c.c. $n/10$ HCl; 1 c.c. $n/10$ HCl contains 3·65 mg. HCl.

Therefore 100 c.c. gastric contents contain $65 \times 3{\cdot}65$ mg. HCl = 237·25 mg. HCl = 0·237 gm. HCl.

Result.—The total acidity of the gastric contents is represented by 0·237 per cent. HCl.

Experiment 161. Estimation of Free Acidity.—10 c.c. of filtered gastric contents are placed in a beaker by means of pipette. Add two or three drops of Töpfer's indicator. The solution turns red. Titrate with $n/10$ alkali until the

red colour is replaced by a lemon-yellow colour. (If the lemon-yellow colour appears at once when the indicator is added, free acid is absent.)

Take burette reading. Record the result.

Since Töpfer's indicator reacts only to free acid which is not combined with protein, the result indicates the amount necessary to neutralise the free acidity of 10 c.c. of the gastric contents.

Calculation is carried out as above.

Explain the results obtained. Refer to Experiment 100.

Experiment 162. Estimation of Total Amount of Hydrochloric Acid Secreted.—To 20 c.c. of the filtered gastric contents apply Volhard's method for the estimation of chlorides as given in Experiment 159, and express the result in terms of HCl.

By deducting the "free acidity" from the "total acidity" the amount of "HCl combined with proteins and organic bases" is arrived at.

By deducting the "total acidity" from the total HCl secreted the amount of "HCl combined with inorganic bases" is arrived at.

Collect the results obtained from Experiments 160, 161, and 162 in tabular form as under :—

<table>
<tr><td rowspan="3">Total HCl secreted by gastric cells :
=</td><td>Free HCl :
=</td><td rowspan="2">Physiologically active HCl :
=</td></tr>
<tr><td>HCl combined with proteins and organic bases :
=</td></tr>
<tr><td colspan="2">HCl combined with inorganic bases :
=</td></tr>
</table>

ABNORMAL CONSTITUENTS OF URINE.

Of the substances which may appear in the urine in pathological conditions, *proteins*, *blood pigments*, *bile pigments and bile salts*, *reducing sugars*, *acetone*, *aceto-acetic acid*, occur most frequently. The fact that these substances are dissolved in urine, a fluid having such a complex composition, introduces certain fallacies in the application of the reactions by which the substances mentioned can be recognised if present alone in a simple watery solution.

In the case of proteins, for instance, the fact that the urine has a colour of its own excludes the use of the colour reactions (biuret, xanthoproteic, etc.) as tests for the presence of proteins in urine. The guaiac test for blood is given also by pus, and a variety of other substances which may occur in urine. In the case of sugar it has been pointed out already that Fehling's solution may be reduced by other substances than glucose or lactose. In order to avoid these fallacies, as many different tests as possible should be carried out before deciding whether a certain substance is or is not present in the urine.

Some drugs are excreted in the urine, and may introduce further fallacies. The previous treatment of the patient must therefore also be taken into consideration.

The following tests for abnormal constituents should be carried out at first on normal urines, then on normal urines to which a very small amount of the abnormal constituents has been added; lastly, on pathological urines obtained from the infirmary.

PROTEINS.

The proteins of the blood plasma (globulin, albumin, fibrinogen) are the proteins most frequently met with in the urine. Clinically no distinction is drawn between

these different proteins, and the presence of any of them is spoken of clinically as "albuminuria"

Tests for Protein in Urine and their Fallacies.

Experiment 163. Tests for Protein in Urine.—For all the following tests the urine must be quite clear. If not clear, filter.

(*a*) *Heat Test.*—The urine must be neutral or very faintly acid. Heat to boiling point. A precipitate may appear which may consist of either earthy phosphates or protein. Add three drops of dilute acetic acid to the hot solution *and heat again to boil.* If the precipitate be one of earthy phosphates it will dissolve. If a flocculent precipitate remains after the addition of the acid, the presence of protein is indicated.

Fallacies.—If the urine is alkaline when heated, or if too much acid is added, alkali albumin or acid albumin is formed, which is not coagulated by boiling and remains in solution. A small amount of protein will therefore not be indicated by this test, unless it is carried out carefully.

(*b*) *Precipitation by Strong Mineral Acids. Heller's Test.*—Place 2 or 3 c.c. of pure nitric acid in a test tube. Incline the test tube and from a pipette allow the urine to flow slowly down the side, so that it forms a layer above the nitric acid. If albumin is present a white opaque ring appears in the urine at the junction of the two fluids. The test is very delicate, and is given by a dilution of 1 : 50,000. If only traces of albumin are present, the ring may appear only after a minute.

Fallacies.—If concentrated urines are examined by this test, a white ring, usually less defined, may be formed, which may be due to the precipitation of uric acid or urea nitrate. If that is suspected, dilute the urine with three times its volume of water, and repeat the test with the

diluted urine. If the ring was due to uric acid or urea nitrate the ring will not appear with the diluted urine.

After the administration of drugs containing resins a white ring may appear owing to the precipitation of the resins by the acids. This ring will disappear on the addition of alcohol to the urine, while a ring due to protein will persist.

Note.—A coloured ring may also appear at the junction of the acid and urine. This is due to the formation of indigo red in urine rich in indican. It has no relation to the presence of albumin.

(*c*) *Precipitation by Alkaloidal Reagents :—*

Precipitation by Hydroferrocyanic Acid.—Render 5 c.c. of urine distinctly acid with 1 to 2 c.c. of acetic acid. Add drop by drop potassium ferrocyanide. If the urine remains clear no albumin is present. If a precipitate forms protein is present.

Precipitation by Salicylsulfonic Acid.—To 5 c.c. of urine rendered slightly acid add drop by drop salicylsulfonic acid. If protein is present a precipitate forms.

Precipitation by Picric Acid.—Place in a test tube 5 c.c. of picric acid solution. Allow a drop of urine to fall from a pipette into the picric acid. If protein is present a cloud will form round the drop.

Fallacies.—If alkaloids are administered in large doses, for instance quinine, they may be excreted in the urine and form a precipitate with these reagents.

Albumoses in Urine.

Albumoses.—In some pathological conditions it will be found that the heat test is negative, while the other tests give a positive result. Then albumoses are present in the urine. It will be found then that the white ring formed in Heller's test dissolves on heating, and reappears on cooling. This condition of *albumosuria* is rare.

REDUCING SUGARS.

On the Presence of Glucose in Normal and in Abnormal Urine.

The sugar which is most frequently excreted in abnormal amounts is glucose. Normal urine contains a very small amount of glucose. It varies in different normal individuals, and in the same normal individuals at different times, from about 0·05 per cent. to about 0·1 per cent., and may occasionally after a meal exceed the latter figure even in normal individuals. The amount is too small to be detected in the urine by the tests which have been used in the past, and therefore it has become the custom to speak of normal urine as being "sugar free," and to speak of the "presence of sugar in the urine" as a pathological condition. The latter expression then means, strictly speaking, that sugar is present in amounts exceeding the normal limits. It so happens that with urine the customary tests (Fehling's test, Nylander's test) begin to give a slightly positive reaction when this limit is exceeded, so that these tests are a valuable criterion whether the urine is normal with reference to glucose or not.

The excretion of glucose in amounts exceeding the normal

limit is called a *glycosuria*, if there is no alteration in other properties of the urine, *e.g.*, colour, specific gravity, total volume excreted. In certain pathological conditions there is in addition to the glycosuria an excretion of a larger amount of water (*polyuria*). The urine is then pale, and as the result of the presence of glucose in large amounts has a high specific gravity. This combination of a *glycosuria* and a *polyuria* constitutes the characteristic symptom of the disease *diabetes mellitus*.

It is important to realise that the mere presence of glucose in abnormally large amounts does not necessarily indicate that the patient is suffering from *diabetes mellitus*. When testing for glucose in urine it is therefore important always to bear in mind the significance of the presence or absence of a *polyuria* by determining the total volume of urine excreted in twenty-four hours. If that information is not immediately available it can be inferred from the colour of the urine.

Tests for Glucose in Urine and their Fallacies.

Experiment 164. Test for the Traces of Glucose present in Normal Urine. Preparation of Reagent.—The test depends upon the reduction of mercuric oxide in a weakly alkaline solution to metallic mercury. The degree of alkalinity is an important factor, as the test becomes more sensitive but less specific the greater the alkalinity of the reagent. The reagent, which is provided, is prepared as follows : 0·4 gm. mercuric oxide (red or yellow) and 6 gm. potassium iodide are dissolved in 100 c.c. water. This solution is weakly alkaline. The alkalinity must now be so adjusted that 10 c.c. of the reagent are neutralised by 2·5 c.c. of $\frac{N}{10}$ acid, using

phenolphthalein as an indicator. This is done by titrating 10 c.c. of the reagent with $\frac{N}{10}$ acid, and, after the alkalinity of the reagent has thus been determined, adding the requisite amount of $\frac{N}{10}$ acid or alkali to the bulk of the reagent. The reagent is a clear, colourless solution which turns slightly yellow on heating, and becomes colourless again on cooling. It must remain quite clear on boiling.

Application of Test.—Apply first this test to a 2 per cent. and 0·2 per cent. aqueous solution of glucose as follows:—5 c.c. of the reagent are heated in a test tube to boiling. The solution remains clear, but turns slightly yellow. 0·5 c.c. (= 10 drops) of the sugar solution is now added, and the mixture again heated to boiling. The test tube is then removed from the flame. The mixture darkens, and a black precipitate of finely-divided mercury appears. At first this precipitate gives the appearance of a turbidity, especially if the amount of sugar present is small. On standing, the precipitate soon settles at the bottom of the test tube.

The test is positive with sugars which give the other reduction tests such as lactose, maltose, xylose, and arabinose. It is negative with cane sugar. The solution is not reduced by uric acid and creatinin, so that the presence of these substances in urine do not introduce a fallacy as in Fehling's test (see Exp. 147, 152).

If the test is applied to urine the resultant turbidity is due not entirely to the precipitated mercury but also to the phosphates which are precipitated when the alkaline reagent is mixed with the hot urine. The turbidity due to precipitated phosphates can be excluded simply by acidifying the mixture, after the test has been carried out, with

acetic acid, which dissolves the phosphates. With normal urines a slight but distinct turbidity remains after the addition of acetic acid, indicating the presence of traces of glucose. The test enables one to recognise not only the presence of small amounts of reducing sugars, but also slight differences in the amounts of sugar present, when these amounts are small. In order to do so, it is necessary to carry out the test in such a way that the conditions under which reduction is produced are constant. Since the addition of acetic acid stops the reduction which would otherwise proceed in the hot mixture, it is necessary to add the acid after a fixed interval, say thirty seconds. As further the precipitate of mercury is heavy and rapidly falls to the bottom of the test tube, the degree of turbidity must be judged at once by holding the test tube over ordinary print. With normal urines print can be read with ease through the mixture.

Application of Test to Urine.—The test then, as applied to urine, which must of course not have undergone ammoniacal fermentation, is as follows:—5 c.c. of the reagent are heated to boiling in a test tube (of ordinary dimensions). The reagent must remain clear. 0·5 c.c. (= 10 drops) of urine is added and the mixture again heated to boiling. The test tube is removed from the flame, and, after thirty seconds, the mixture is acidified with a few drops of acetic acid. The test tube is at once held over ordinary print. If the urine is normal, a slight but distinct turbidity remains, and print is clearly readable through the mixture.

If the turbidity is more intense and print no longer clearly readable through the mixture, the amount of sugar present distinctly exceeds the normal, and the other tests for glucose given in the following experiment will then give positive results.

When only small quantities of sugar are present it is easier

to judge roughly the amount of sugar present from the degree of turbidity obtained in this test than from the changes observed in Fehling's or Nylander's test. The test is particularly useful when the amounts of sugar present exceed the normal amount only slightly; that is to say, in those conditions where Fehling's test gives ambiguous or doubtful results.

Experiment 165. Tests for Abnormal Amounts of Glucose in Urine.—(*a*) *Trommer's Test.*—To 5 c.c. of urine add 1 c.c. of caustic soda. Then add, drop by drop, cupric sulphate, shaking after the addition of each drop. On the addition of each drop a flaky precipitate of cupric hydrate forms at first, which, if sugar is present, dissolves on shaking, giving a deep blue solution. Continue to add cupric sulphate, drop by drop, until a little cupric hydrate remains undissolved. Heat the upper part of the solution. A yellow precipitate forms if a reducing sugar is present.

(*b*) *Fehling's Test.*—Heat in two test tubes equal volumes of urine and of Fehling's solution to boiling point. The Fehling's solution must remain clear on boiling. Otherwise it has become decomposed, and a fresh solution must be prepared. When both solutions are boiling, remove the test tubes from the flame and pour the one into the other without further heating. Allow the mixture to stand. If much sugar is present yellow cuprous oxide separates out at once. With less sugar present the colour gradually changes. This is followed by the appearance of a yellow precipitate, which may not begin to appear until the tube has cooled.

If little sugar is present repeat the test with Fehling's solution half diluted with water.

Note.— Although the principle of Trommer's and Fehling's tests is the same, it is best to use both in testing for reducing sugars, as there are certain advantages and disadvantages connected with either of them.

Fallacies.—The fallacies due to the presence of uric acid and creatinine have already been discussed (see Exps. 147, 152). Further:—

Glycuronic Acid.

Glycuronic acid reduces cupric salts. This acid may occur in the urine as the result of administration of certain drugs, such as chloral, morphia, camphor, chloroform, antipyrin, antifebrin, or it may occur as the result of excessive intestinal putrefaction. (Explain its appearance in both these conditions.) If the test for indican shows that a large amount of indican is present, it points to the latter possibility. Glycuronic acid (what is its structural formula?) can be distinguished from glucose by its failure to ferment with yeast.

In connection with the use of chloroform as a preservative for urines, it must also be borne in mind that chloroform reduces cupric salts, the mixture turning first red and then dark brown, owing to the reduction of cupric salts to colloidal cuprous oxide (red) and colloidal copper (black).

(*c*) *Nylander's Test.*—To some urine in a test tube (wide tubes are most convenient) add one-tenth of its volume of Nylander's reagent. Boil over a small flame for three minutes and allow to cool. If reducing sugar is present the urine darkens to a deep brown, and eventually a fine black precipitate settles out. Normal urine shows only a slight darkening.

Fallacies.—The same as in Trommer's or Fehling's test, except that uric acid and creatinine do not reduce Nylander's reagent. On the whole this test is more reliable than Fehling's or Trommer's test.

(*d*) *Fermentation.*—For this test the urine must be clear and as fresh as possible. It must not have entered into ammoniacal fermentation. Test reaction. If alkaline,

make faintly acid with a few drops of very dilute acid (tartaric acid). Boil the urine for two minutes and cool under the tap. Place a small piece (size of a pea) of yeast in the cooled urine in a test tube and shake up gently, so that an emulsion of yeast in the urine is formed. Fill the fermentation tube with this emulsion, so that the closed limb contains no air bubbles. Allow the tube to stand in a warm place (best at 37°), and examine after one hour, and again after twenty-four hours. The appearance of a bubble (of CO_2) in the closed limb indicates the presence of glucose.

Fallacies.—This is the most reliable and important test for glucose in the urine, since glucose is the only fermentable substance which may occur in the urine. The only possible fallacies arise from possible irregularities in the behaviour of the yeast. Firstly, the yeast may be inactive, so that no fermentation takes place although glucose is present. Secondly, the yeast may undergo self-fermentation, that is, it may give off gas, even in the absence of glucose. These two fallacies can be excluded by the following two control experiments, carried out at the same time with the same yeast.

One tube contains an emulsion of yeast in a dilute glucose solution. Absence of fermentation in this tube would indicate that the yeast is inactive, and therefore useless.

The other tube contains an emulsion of yeast in normal urine. If more than a very small air bubble is found in the closed limb of this tube the yeast undergoes self-fermentation, and the results obtained are not reliable.

(*e*) *Phenylhydrazine Test.*—To 10 c.c. of urine add ten drops of phenylhydrazine and equal amount of glacial acetic acid. Shake the test tube to ensure a thorough mixing, and keep in the *boiling* water-bath for one hour. Allow to cool slowly. If crystals separate out, examine

microscopically. If glucose is present the characteristic crystals of the glucosazone should be seen. If only small amounts of glucose are present this test may be negative.

Note.—The value of this test is that it enables one to distinguish glucose from other reducing sugars, especially lactose, which may be present. The other sugars also form osazones, which, however, have a different crystalline form.

(*f*) *Polarimetric Examination.* — If a polarimeter is available the urine should be examined with regard to its action on polarised light. If glucose is present, the urine will produce dextrorotation. If the urine is too deeply coloured it should be decolorised by shaking with solid lead acetate and filtering until the filtrate is clear.

Lactose, its Distinction from Glucose.

Lactose is sometimes present in the urine. It gives the same tests as glucose, but can be distinguished from it by the form of the osazone crystals and by its inability to ferment.

Method of Testing Urine for Reducing Sugars.—Proceed as follows: Note the colour of the urine and its specific gravity. If proteins are present they must be removed by heat coagulation before testing for sugars. Apply mercuric oxide test. Judge from "print-reading" or by comparison with a normal urine whether the amount of sugar present is normal, slightly in excess, or abundant. Note the result. Then apply Trommer's or Fehling's test. If the mercuric oxide test gives a normal reading and the cupric oxide test are undoubtedly negative, the urine is normal with reference to reducing sugars, and no further tests are necessary. If the tests are distinctly positive, the phenylhydrazine test and fermentation test must be carried out to identify the reducing sugar present and to exclude all possible fallacies. A polarimetric examination is desirable. If the cupric oxide tests give a doubtful result, the reduction may be due either to substances other than reducing sugars, or it may be due

to the amount of sugar present being only slightly in excess of the normal. This can be decided by reference to the result of the mercuric oxide test and confirmed by Nylander's test. In the latter case an attempt should be made to identify the sugar present with the phenylhydrazine test.

Quantitative Estimation of Glucose.

Experiment 166. Titration of Glucose by Fehling's Method.—This titration is based on the principle that a definite amount of copper salt, in this case the amount contained in 10 c.c. of Fehling's solution, is reduced to the red cuprous oxide by a constant amount of glucose (0·05 gm. glucose). When all the blue copper sulphate has been reduced to red cuprous oxide, which falls out, the end-point of the reaction is reached, and the blue colour of the supernatant fluid, which is due to the presence of copper sulphate, totally disappears. The volume of urine used is then known to contain 0·05 gm. glucose.

Method.—Run exactly 10 c.c. of Fehling's solution from a burette into a porcelain basin, dilute with about 40 c.c. of water, and heat to boiling. While the Fehling's solution is kept boiling gently, run in the urine from a burette. Add $\frac{1}{2}$ c.c. of the urine at a time in short intervals. A red precipitate appears suspended in a fluid which is, at first, deep blue. As more and more of the urine is added the blue colour of the fluid in which the precipitate is suspended becomes fainter and fainter. Finally the red precipitate will be seen suspended in a fluid which is colourless (or slightly yellow). The point when the fluid, in which the precipitate is suspended, first becomes colourless indicates the end-point of the titration.

The first titration gives only a rough estimate of the amount of urine necessary to reduce 10 c.c. of Fehling's solution. If the urine contains so much glucose that less than 5 c.c. of urine have been used in this first titration.

the urine must be diluted until about 6 to 12 c.c. of urine are required to arrive at the end-point. (If, for instance, 2·8 c.c. of urine have been used in the first titration, the urine should be diluted three times, *i.e.*, 20 c.c. of urine with 40 c.c. of water.) Empty the burette containing the original urine, wash out with the diluted urine, and then fill with the diluted urine.

In order to determine the end-point accurately, subsequent titrations are carried out in such a way that instead of adding the urine in small portions, almost all the urine necessary to produce complete reduction is added in bulk to the Fehling's solution. Continue boiling after the addition, and complete the reaction until the supernatant fluid is colourless by adding the urine in small quantities (three drops at a time). The end-point is best seen if, after allowing the red precipitate to settle, the basin is slightly tilted so that the supernatant fluid appears against the white background of the porcelain.

Carry out one preliminary and two subsequent titrations. If the two subsequent titrations agree to within 0·5 c.c., take their average, and calculate from it the amount of sugar present in 100 c.c. of urine.

Note.—The fallacy to guard against in this titration is the oxidation by air. If, for instance, a titration has been completed, and the red precipitate with the supernatant colourless fluid is allowed to stand in contact with the air, a blue colour will again appear in the supernatant fluid owing to oxidation and solution of the cuprous oxide. If, therefore, the end-point of the reaction has once been reached, subsequent appearance of a blue colour must be neglected.

For the same reason the whole titration should be carried out as quickly as possible.

Calculation. Example :—

Preliminary titration. 10 c.c. Fehling are reduced by 3·3 c.c. of urine. Dilute 20 c.c. of urine with 40 c.c. of water. Fill burette with urine thus diluted, 1 : 3.

First titration. 9·1 c.c. urine.

Second titration. 8·8 c.c. urine.

Average, 8·95 c.c. urine.

8·95 c.c. diluted urine contain 0·05 gm. glucose.

100 c.c. diluted urine contain $\frac{0{\cdot}05 \times 100}{8{\cdot}95}$ gm. glucose = 0·56 gm. glucose.

Result.—*The original urine was three times stronger, and contained therefore in* 100 *c.c. urine* 1·68 *gm. glucose.*

From this the amount of glucose excreted in twenty-four hours can, if necessary, be calculated.

Experiment 167. Quantitative Estimation of Glucose by Polarimeter.—The method of using the polarimeter has already been described (see Exp. 16).

Method of Preparing the Urine for Examination by the Polarimeter.—The urine must be free from proteins, since these substances are themselves optically active and lævorotatory. If proteins are present, they must be removed by heat coagulation. If the urine is cloudy, it must be filtered until it is clear. Diabetic urine is, as a rule, only lightly coloured, and can frequently be used without removing the colouring matter. If the urine is coloured to such an extent that, after inserting the polarimeter tube filled with urine, the dividing line between the two halves of the field cannot be seen clearly, the urine must be decolorised. This is done by boiling the urine with some animal charcoal or by shaking it with about one-tenth its bulk of lead acetate and filtering. The urine

is then cooled to room temperature, and the polarimeter tube filled with the urine in such a way that no air bubbles are present. The tube is then placed in position in the apparatus, and the rotation is read to within one-tenth of a degree.

Calculation.—The calculation depends on the fact that every optically active substance has a definite rotatory power. The actual rotation produced by a solution of an optically active substance depends on (1) this "specific rotatory power," (2) the wave-length of the light used, (3) the concentration of the solution, and (4) the length of the column of fluid which the polarised light traverses. In the case of glucose, and with the light used in an apparatus of the Soleil pattern, a glucose solution which contains 1 gm. of glucose in 100 c.c. of water rotates polarised light through 0·59°, when the light traverses a column of glucose solution 100 mm. long. If the column of glucose solution is twice as long—200 mm.—the rotation is twice as great, namely, 1·18°. In all quantitative polarimetric estimation the length of the tube which contains the glucose solution must therefore be known.

If a 200 mm. polarimeter tube is used the percentage p of the glucose solution is found by dividing the actual rotation α, which has been observed, by 1·18.

$$p = \frac{\alpha}{1{\cdot}18}.$$

This formula applies to a tube 200 mm. long. With a tube half that length the result has to be doubled.

$$p = \frac{2\alpha}{1{\cdot}18} = \frac{\alpha}{0{\cdot}59}.$$

Note.—Since with a 200 mm. tube a 1 per cent. glucose solution produces a rotation of 1·18°, each degree of rotation corresponds to 0·84 per cent. of glucose. By

using a slightly shorter tube, 168 mm. long, it is possible, therefore, to obtain readings so that 1° of rotation corresponds to 1 per cent. of glucose. In other words, the degree of rotation indicates directly the percentage of glucose. Instruments for clinical use are frequently arranged in this way.

ACETO-ACETIC ACID AND ACETONE.

These two substances always occur together, since acetone is formed by decomposition from aceto-acetic acid. What are the structural formulæ for these two substances?

The appearance of these two substances indicates a severe metabolic disturbance to which the name *acidosis* has been given. It is frequently associated with other pathological conditions, such as diabetes mellitus, chloroform-poisoning, starvation, withdrawal of carbohydrates, etc.

ACETO-ACETIC ACID.

Note.—In testing for aceto-acetic acid the urine must be quite fresh, as aceto-acetic acid is easily decomposed on standing into acetone.

Note the odour.

Tests for Aceto-Acetic Acid.

Experiment 168. Nitroprusside-Ammon.-Sulphate Test (*Rothera's Modification*).—Take 10 c.c. of urine in a test tube and saturate it with ammonium sulphate by shaking it with an excess of the solid salt. Then add two or three drops of a freshly prepared solution (5 per cent.) of sodium nitroprusside. Add 2 or 3 c.c. of strong ammonia. Mix by inverting the test tube once or twice. Allow to stand undisturbed for twenty minutes. If aceto-acetic acid (or acetone) is present a deep permanganate colour appears The rapidity with which the colour develops and the depth

of the colour give a rough measure of the amount of aceto-acetic acid (and acetone) present.

The test is very sensitive, and is given by aceto-acetic acid even in concentrations of 1 : 200,000 and less.

Repeat the test with normal urine. Note that a faint reddish colour is obtained.

Repeat the test with urines containing decreasing amounts of aceto-acetic acid. These can be prepared by mixing a urine from a case of acidosis with normal urine in the proportions 1 : 2, 1 : 5, 1 : 10, 1 : 100.

Experiment 169. Ferric Chloride Test. (*Gerhard's Test*).—To 5 c.c. of urine add, drop by drop, ferric chloride as long as a precipitate of ferric phosphate continues to form. A claret-red colour is produced if sufficient diacetic acid is present.

If, owing to the presence of the ferric phosphate precipitate, the colour is difficult to recognise, filter. This test is much less sensitive than the nitroprusside ammon.-sulphate test, and is given only when the concentration of aceto-acetic acids is 1 : 1,000 or higher.

Fallacies.—Carbolic acid, salicylates, antipyrin, etc., give a similar colour. They can be distinguished from diacetic acid, because the colour produced by the latter disappears on boiling, while the colour produced by the drugs named persists.

Why is the colour produced by diacetic acid destroyed by boiling?

Diacetic acid may be extracted from urine acidified with hydrochloric or sulphuric acid, by shaking with ether. Remove the ether with a pipette, and add to the ether ferric chloride. A claret-red colour appears if diacetic acid is present.

ACETONE.

Note the odour.

Unspecific Test for Acetone.

Experiment 170. Nitroprusside-Ammon.-Sulphate Test.—Carry out the test as described in Experiment 167. The same result is obtained.

The test is given both by aceto-acetic acid and by acetone, but is not so sensitive for acetone as for aceto-acetic acid. It is given by acetone only when the concentration is 1 : 20,000 or higher.

METHOD OF EXAMINING THE URINE IN CASES OF ACIDOSIS.

Much significance was attached formerly to the question whether acetone alone was present, or whether both acetone and aceto-acetic acid were being excreted. It was believed that the latter condition represented a more severe disturbance of metabolism than the excretion of acetone alone. This view is, however, erroneous. It probably arose from the fact that the only test formerly available for aceto-acetic acid, the ferric chloride test, is not so sensitive as the tests formerly used for acetone.

An indication of the severity of the disturbance is given by the amounts of abnormal acids formed. This can be determined directly by estimating by means of special methods the amount of acetone and diacetic acid. It can be determined indirectly by determining the "ammonia coefficient" (see page 92), since the organism responds to an increased formation of acid substance by an increased excretion of ammonia and a diminished excretion of urea.

So far as the qualitative examination of the urine is concerned, no great significance attaches to the question

whether acetone alone or aceto-acetic acid alone is present. It is therefore sufficient to apply first the nitroprusside-ammon.-sulphate test. If this test is negative, no further tests are necessary. If the test is positive, some indication of the amounts present is given by (1) the intensity of the colour and (2) the rate at which it develops. Some further indication of the degree of acidosis may be obtained by applying the ferric chloride test, which is positive only with a concentration of aceto-acetic acid exceeding 1 : 1,000. Note that this test must be applied to freshly voided urine.

It is not of great importance to apply separate tests for acetone. If aceto-acetic acid is present, acetone will always be present too, especially if the urine has been allowed to stand, since aceto-acetic acid is readily decomposed with the formation of acetone. It may be of interest to determine whether in freshly voided urine acetone is absent or present. In that case the following test, which is very sensitive, may be applied.

Specific Test for Acetone.

To 5 c.c. of urine add 1 c.c. of a 10 per cent. solution of salicylaldehyde in alcohol. Mix by shaking gently. Add a piece of a solid caustic soda stick (or caustic potash stick) about one inch long. Allow to stand undisturbed. If acetone is present a deep brownish-red colour develops at the point of contact.

BLOOD PIGMENT.

Tests for Blood in Urine.

Experiment 171.—(*a*) *Colour.*—If a relatively larger amount of blood is present the urine has a red colour. Smaller quantities give to the urine an opaque reddish brown appearance, "smoky urine."

Examine microscopically the deposit, if any, for the presence of red blood corpuscles.

(*b*) *Colour of Earthy Phosphate Precipitate.*—Render 5 c.c. of urine strongly alkaline with caustic soda, and boil. A precipitate of earthy phosphates will separate out on standing. This precipitate is normally greyish white. If blood is present it is brownish red in colour from the hæmatin which is carried down with it.

Fallacies.—Cascara sagrada, rhubarb, senna, give to the precipitate a similar coloration.

(*c*) *Guaiac Test.*—To 5 c.c. of urine add 1 c.c. of hydrogen peroxide and shake. Then add two drops of tincture of guaiac, so that the resin floats on the urine. A blue colour appears at the junction of urine and resin if blood is present. This test is a very sensitive one.

Fallacies.—Iodides and pus also give a blue colour.

The presence of pus can be excluded by previously boiling the urine. If the guaiac is then applied while the urine is still hot, the test becomes even more sensitive.

(*d*) *Spectroscopic Examination.*—Examine urine spectroscopically as described in Experiments 76 to 83. If blood is present the spectrum of methæmoglobin is usually seen. This may have been formed from hæmoglobin on standing after the urine has been passed, or it may have been passed as such.

If the absorption spectrum is not distinct, prepare hæmochromogen by boiling with caustic soda and reducing with ammonium sulphide (see Exp. 81). If hæmoglobin or methæmoglobin are present the characteristic spectrum of hæmochromogen will be seen.

Spectroscopic examination is free from fallacies, but it does not indicate traces of blood as the guaiac test does. It is therefore possible that the guaiac test is positive, while spectroscopic examination is negative.

BILE.

Tests for Bile Constituents in Urine.

Experiment 172. Bile Pigments.—(*a*) *Colour.*—If bile pigments are present the urine has a brownish or greenish colour. The latter colour is present especially if the urine has been standing for some time, so that the red bilirubin has become oxidised to the green biliverdin.

The chemical tests for bile pigments depend on the oxidation of the red bile pigment to the green bile pigment, so that the appearance of a green colour after the application of an oxidising agent indicates the presence of bile pigments. In *Gmelin's test* (see Exp. 123) the oxidation is carried out with strong nitric acid, in *Huppert's test* (see Exp. 124) with ferric chloride after the pigment has been carried down with a precipitate of calcium carbonate or barium carbonate, to which it adheres, and from which it is extracted by acid alcohol. Apply both these tests to urine, following the instructions given in Exp. 123 for *Gmelin's test*, and Exp. 124 for *Huppert's test*. *Gmelin's test* is best carried out in the last modification described on p. 66, in which the urine is filtered repeatedly through a filter paper, and a drop of nitric acid is then applied to the paper. This test is not very delicate. *Huppert's test* is more delicate. A very reliable and convenient test is the

Maréchal-Smith's Iodine Test.

This test, in which the oxidation is carried out by means of an alcoholic solution of iodine is performed as follows: Place 5 c.c. to 10 c.c. of urine in a test tube. Incline the tube until it is nearly horizontal, and allow one or two drops of the 2·5 per cent. tincture of iodine to trickle down the side of the tube, so that it does not mix with the urine but forms a layer on top of it. Hold the tube against

a white background. If bile pigments are present the red iodine layer on top and the yellow urine below are separated by a green layer. The urine and the iodine solution should not be allowed to mix, and an excess of iodine must be avoided. If the urine is very darkly coloured it should first be diluted with water.

Experiment 173. Bile Salts.—The identification of bile salts in the urine is not of any great practical importance, since they are always associated with bile pigments. *Pettenkofer's test* (see Exp. 122) applied to urine is not reliable, since urine contains other substances which give colour reactions with sulphuric acid. In the modification described on p. 66, Note 3, apply the test to normal urine and to urine containing bile. A better test, *Hay's test*, by which the presence of bile salts can be recognised, depends on the fact that bile salts diminish the surface tension of the solution in which they are present (see Exp. 119). Flowers of sulphur sprinkled on normal urine float, but will sink if the urine contains bile salts.

SOME PATHOLOGICAL DEPOSITS.

Morphological elements, such as pus cells, epithelial cells, casts, etc., are not dealt with here.

Cystin.—Hexagonal plates, insoluble in water and acetic acid, soluble in hydrochloric acid and in ammonia.

Leucin and Tyrosin.—Usually occur together, especially in diseases of the liver.

Leucin is deposited in spheres having a radial and concentric striation. It is slightly soluble in water. Soluble in acids and alkalis.

Tyrosin is deposited in sheaves of fine white needles. The appearance of the crystals is very much like that of glucosazone, except that the latter is yellow. Tyrosin is very slightly soluble in water, soluble in acids and alkalis. It gives a red colour with Millon's test.

What are the structural formulæ for cystin, leucin, and tyrosin? What are they formed from?

Calcium Oxalate.—See under normal urines (page 73). Is pathological only if present in excess.

Experiment 174.—Examine urines of cases from the infirmary for pathological constituents, and report on the form given out.

Experiment 175.—Examine urines of cases from the infirmary for total N, urea, ammonia, acidity, chlorides, sulphates, indican, and report on the form given out.

FORM FOR REPOR

Name of Patient	
Diet and Treatment (Drugs) during previous twenty-four hours	
Total volume of urine excreted in twenty-four hours	
Colour	
Deposit, if any	
Specific gravity	
Reaction	
Total N excreted in twenty-four hours	
Urea. Amount excreted in twenty-four hours	
,, Percentage of urea N to total N	
Ammonia. Amount excreted in twenty-four hours	
,, Percentage of ammonia N to total N	
Acidity of total urine	
Chlorides. Amount excreted in twenty-four hours	
Sulphates[1]	
Ethereal sulphates[1]	
Indican[1]	

1 c.c. $\frac{n}{10}$ acid = 1·7 mg. NH_3 = 1·4 mg. N; 1 c.c. standard $AgNO_3$ = 10 mg liberates 354 c.c. N.

[1] State by + or − whether test is positive or negative. A doubtful result

[2] State here your general conclusion; for instance: "Glucose absent

ON URINE ANALYSIS.

Abnormal Constituents.			
Test.		Result.[1]	Conclusion.[2]
Albumin.	Heat		
	Nitric acid		
	Picric acid		
	Hydroferrocyanic acid		
	Salicylsulfonic acid		
Reducing sugar.	Mercuric oxide		
	Fehling		
	Fermentation		
	Nylander		
	Osazone		
	Quantitative estimation		
Blood.	Guaiac		
	Earthy phosphates		
	Spectroscopic examination		
Aceto-acetic-acid.	Nitroprusside - ammon. sulph.		
	Ferric chloride		
Acetone.	Nitroprusside-ammon.-sulph.		
Bile.	Gmelin		
	Huppert		
	Iodine		

NaCl; 10 c.c. standard Fehling's solution = 50 mg. glucose; 1 gm. urea

may be indicated by + ?, a strong positive result by + +, and so on.
although Fehling's and Trommer's slightly positive."

THE BALANCE BETWEEN ACIDS AND BASES IN THE ORGANISM.

The organism has the power to establish and maintain a balance between acids and bases, so that the reaction of the tissue fluids remains always near the neutral point, *i.e.*, that of distilled water, but slightly on the alkaline side. It does so notwithstanding the fact that inorganic acids, such as carbonic acid, phosphoric acid, and organic acids are constantly being formed in the course of metabolism and poured into the blood stream. The neutral reaction is maintained even when acids are being formed in excess—for instance, lactic acid, or when abnormal acids, such as oxybutyric acid and aceto-acetic acid, are making their appearance. The term acidosis, which is applied to the latter condition, means, therefore, only that abnormal acids are being formed, but does not imply that the reaction of the blood becomes acid. The power to maintain a neutral reaction of the tissue fluids is due chiefly to the presence therein of the carbonates and phosphates of sodium: solutions of such salts of strong bases and weak acids behave in such a way, when acids are added to them, that the effect of the added acid, so far as the reaction of the fluid is concerned, is much less pronounced than if the same amount of acid had been added to water alone or to a solution of a salt of a strong base and a strong acid such as sodium chloride. These salts may therefore be said to act as

"dampers" or "buffers" against the added acid. This effect is illustrated in the experiments given below, which demonstrate also the rationale of the use of indicators.

The organism rids itself eventually of the acid substances which it produces by excreting them. The important point to be noted is that it does so without altering the neutral reaction of its tissue fluids. The excretion of the acid products just referred to is effected chiefly in three ways: (1) the excretion of CO_2 by the lungs; (2) the excretion of acid phosphates by the kidneys; (3) by using the ammonia which is also formed as a product of metabolism, to combine—(*a*) with CO_2 to form urea to be excreted by the kidneys; (*b*) to combine with other acids and form neutral ammonium salts, which also are excreted by the kidney.

The following theoretical considerations may briefly be recalled.

Definition of Acidity and Alkalinity.—*Acids can be defined as substances which when dissolved in water furnish free H-ions; bases as substances which in watery solution furnish free OH-ions.* The acidity of a solution is thus due to the presence of an excess of H-ions, alkalinity to the presence of an excess of free OH-ions. Thus HCl is dissociated into H-ions and Cl-ions, acetic acid into H-ions and CH_3COO-ions, sodium hydroxide into Na-ions and OH ions. The extent to which the various bases and acids are dissociated is, however, not the same. Some acids, such as HCl, are almost completely dissociated into their ions, others, such as acetic acid, are only partially dissociated. Thus it has been found that in decinormal HCl 84 per cent. of the available H is present in the form of free H-ions. Completely dissociated acids are "strong" acids, partially dissociated acids are "weak" acids.

When a solution of an acid is being neutralised by the

addition of an alkali, free OH-ions are added to the solution containing free H-ions. These form water, which is only slightly dissociated. At the neutral point there is equality in the number of free H- and OH-ions, the concentration of these two corresponding to that present in pure water.

Notation of Hydrogen-ion Concentration.—Acidity can thus be expressed in terms of the concentration of H-ions by stating the weight of free H-ions present in a litre of solution. This is stated in terms of normal solutions, a N/1 solution being one which contains 1 gm. H-ions in 1,000 c.c. A normal solution of HCl contains 36·5 gm. of HCl in 1,000 c.c. of water, of which 1 gm. is hydrogen. If in that solution the acid were completely dissociated into H-ions and Cl-ions, then the H-ion concentration in a N/1 HCl solution would be N/1. Similarly that of a N/10 HCl solution would be N/10, that of a N/1,000 HCl solution would be N/1,000. It has been found experimentally that these various HCl solutions are not completely dissociated into H-ions and Cl-ions; a small percentage remains undissociated as HCl, so that, for example, the H-ion concentration of a N/10 HCl solution is slightly less than N/10.

Instead of using the fractions N/10, or 0·1 N, N/100 or 0·01 N, N/1,000 or 0·001 N, it has been agreed to use the exponential form, so that $0{\cdot}1 = 10^{-1}$, $0{\cdot}01 = 10^{-2}$, $0{\cdot}001 = 10^{-3}$.

This has been further abbreviated by using the exponent only, omitting both the base 10 and the negative sign. This is called the "Hydrogen-ion Exponent," and written P_H. Thus $P_H = 1$ means the concentration of a N/10 solution of H-ions; $P_H = 3$ means the concentration of a N/1,000 solution of H-ions. Hence the practical rule: *As the* P_H *decreases the H-ion concentration increases*, i.e., *the solution becomes more acid.* For example, $P_H = 3$ represents a

more acid solution than $P_H 4$. Further, a difference of 1 in the P_H, as in the example just quoted, represents a ten-fold difference in the concentration. $P_H = 3$ indicates that the solution contains ten times as many H-ions as a solution with $P_H = 4$.

Neutrality.—This can be expressed in terms of H-ion concentration by using the P_H of pure water. Since the H-ion concentration of pure water has been found to be 10^{-7},* $P_H = 7$ indicates neutrality. In water the OH-ion concentration is the same as the H-ion concentration.

Alkalinity.—Instead of expressing increasing alkalinity in terms of increasing concentration of OH-ions, it can be expressed in terms of diminishing concentration of H-ions. Since $P_H = 7$ indicates the neutral point, and all values below 7 indicate acidity, it may thus be looked upon as a zero point, from which the values for P_H fall away as the acidity increases and rise as the alkalinity increases.

The Indication of the H-ion Concentration by Indicators.—The H-ion concentration can be determined directly by a physical method. For most purposes an indirect method by indicators is sufficient.

Experiment 176. Indicators are Substances which Change their Colour or Shade with Variations in the H-ion Concentration.—Prepare 2N, N, 0·1 N, 0·01 N, 0·001 N solutions of HCl. Place 10 c.c. of each into five test tubes. Add five drops of a 0·05 per cent. crystal-violet solution. Note that to each strength of acid corresponds a different colour.

The experiment may also be carried out as follows:—Place 10 c.c. of the 2N acid into a beaker. Add ten drops of crystal-violet. The colour is greenish. Now add water.

* The exact value is $10^{-7\cdot07}$.

Tabulate the results as follows :—

Indicator.	0·1 N HCl.	0·1 N Acetic Acid.
Phenolphthalein -		
Phenol red - -		
Methyl red - -		
Methyl orange - -		
Töpfer's reagent -		

The experiment shows :—

(1) That different indicators require different amounts of the same acid to reach their "turning point," *i.e.*, their "turning point" lies at different H-ion concentrations.

(2) That with a strong and a weak acid the same amount is required to neutralise alkali and reach absolute neutrality (the turning point of phenol red), but that from that point on, larger quantities of the weak acid than of the strong acid are needed in order to increase the H-ion concentration. This illustrates again the difference between titration acidity and H-ion concentration, which has been demonstrated in Experiment 177.

The "Buffer Effect."

Experiment 180. Salts of Strong Bases and Weak Acids act as Buffers for Free H-ions.—Place 10 c.c. of 0·1 N NaOH in a beaker containing 10 c.c. of water. Add five drops of phenolphthalein and titrate with 0·1 N HCl until the red colour of phenolphthalein just disappears. This will require 10 c.c. of the 0·1 N HCl, and brings the solution near the point of absolute neutrality, so that it is now a solution of NaCl. Now add to the solution five drops of Töpfer's reagent or of methyl orange, and continue adding decinormal HCl drop by drop until the turning point is reached. Note the amount of decinormal HCl

necessary to reach from the neutral point the H-ion concentration necessary to turn these indicators.

Repeat the experiment, but neutralise the 10 c.c. of decinormal NaOH with decinormal acetic acid instead of hydrochloric acid, until the red colour of phenolphthalein has disappeared. The solution is now a neutral solution of sodium acetate. Now add to the solution five drops of Töpfer's reagent, or of methyl orange, and add decinormal HCl until the turning point is reached. Note the amount, which is now very much larger than in the first experiment.

In this experiment decinormal HCl has been added in the one case to a neutral solution of sodium chloride, in the other case to a neutral solution of sodium acetate. The addition of equal amounts of HCl does not produce in the two solutions an equal increase in the H-ion concentration, which increases only slowly as the HCl is added to sodium acetate. Sodium acetate acts as a "damper" or "buffer."

The explanation is as follows: When a strong acid is added to a salt of a strong base and a strong acid no subsidiary reaction occurs. Therefore the H-ion concentration is the same as that obtained by adding a strong acid to water, and increases rapidly with the addition of the strong acid. But if a salt of a strong base and a weak acid is present a secondary reaction occurs. The strong acid combines with the strong base, and the weak acid is liberated. The H-ion concentration of the mixture is now not due to the strong acid which has been added but to the weak acid which has been liberated. And since weak acids are only slightly dissociated into free H-ions, the number of free H-ions in the mixture is much less below the number of free H-ions originally present in the strong acid which was added.

Experiment 181. The Buffer Action of Sodium Bicarbonate.—Place 10 c.c. of a 0·25 per cent. solution of sodium bicarbonate in a beaker with five drops of methyl orange.

Add decinormal HCl until the turning point is reached. Note that in this case the weak acid CO_2 which is liberated is given off to the air. Compare with the amount of acid necessary when water is used instead of the sodium bicarbonate solution.

All salts of strong bases with weak acids such as phosphates, bicarbonates, citrates, acetates, can act as dampers or buffers. Proteins can also act as buffers by combining with the acid. (See Experiment 100.)

Experiment 182. The Buffer Function of the Sodium Bicarbonate in Blood.—Place 20 c.c. of a 0·25 per cent. NaCl solution into one flask, 20 c.c. of 0·25 per cent. solution of $NaHCO_3$ into another. Add to each ten drops of phenol red. Take a deep breath, and by means of a pipette blow the expired air through the NaCl solution. One deep expiration will be sufficient to turn the colour of the indicator from red to yellow. Repeat with the $NaHCO_3$ solution. Note that the red colour becomes pink, but is not turned to yellow, even after several deep expirations. Now agitate the solution so as to aerate it. The solution regains its original intense red colour, because the dissolved CO_2 is given off to the air.

The experiment with $NaHCO_3$ reproduces diagrammatically the condition obtaining in the blood, when the passage of acid metabolites into the blood is accompanied by a comparatively slight increase in the H-ion concentration. This by stimulating the respiratory centre leads to increased ventilation, which removes CO_2 and restores the original H-ion concentration. In this way the tissue fluids can deal with large amounts of acid metabolites without becoming acid. Note that the term acidosis applies to the excessive production of acid metabolites and their passage into the blood, but does not indicate an acid reaction of the blood.

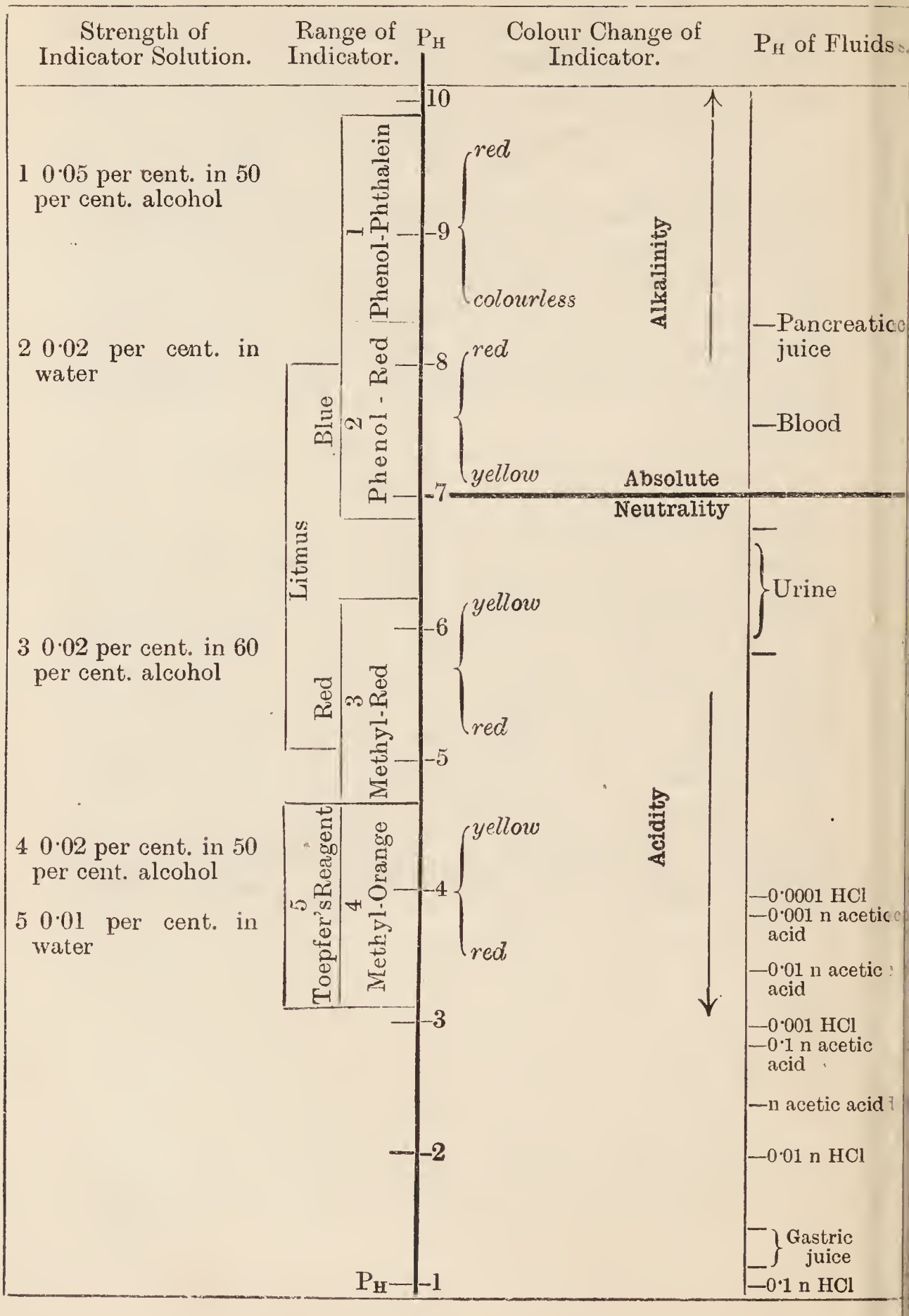
Experiment 183. Approximate Estimation of the Hydrogen-ion Concentration of the Urine.—Place 5 c.c. of freshly voided urine into each of three test tubes. Add to one five drops of methyl orange, to the other five drops of methyl red, to the third five drops of phenol red. If methyl red strikes a yellow colour, the value for P_H is near 6, if a red colour, the value is near 5. With most normal urines methyl orange will strike a yellow colour, phenol red also a yellow colour. If methyl orange gives an orange colour the urine is abnormally acid; if phenol red gives a pink colour the urine is abnormally alkaline. (See Table of Indicators.)

Note that the urine must be freshly voided, so that ammoniacal fermentation is excluded.

In order to make an exact estimation of the H-ion estimations of fluids, such as blood serum, urine, gastric juice, etc., the colour given by 5 c.c. of the fluid with five drops of an indicator of suitable range is compared with the colour given by the same indicator with a number of mixtures of salt solutions, the H-ion concentration of which is known. For a detailed account of the method of preparing these salt mixtures reference may be made to Cole's "Practical Physiological Chemistry," also to the pamphlet, "The Reaction of Media," published by the Medical Research Committee (Special Report Series, No. 35).

TABLE.

Range of H-ion Concentration for Different Indicators.



INDEX

Absorption spectra, 40
Aceto-acetic acid, 114
Acetone, 114, 116
Achromic point, 54
Acids in gastric contents, 57
Acidity,
  definition of, 125
  estimation by H-ion concentration, 128, 133
  estimation by titration, 84, 128
  of urine by titration, 87
  of gastric contents by titration, 96
Acidosis, 93, 116, 124
Albumin,
  in egg-white, 15
  in milk, 20
  in blood-serum, 46
  in urine, 99
  heat, coagulation of, 15
  properties of (table), 61
  removal of, 49
  solubilities of (table), 48
Albumoses,
  detection of, 60
  formation of, 59, 63
  in urine, 102
  properties of (table), 61
Alcohol, 3
Alkalinity, 125, 127
Ammonia in urine, 76
  estimation of, 87
Amylopsin, 62
Antagonism between Ca- and Na-ions, 69

Bile, 64
  in urine, 119
Biuret test for proteins, 16
Blood,
  coagulation of, 31 37
  laking of, 38
  oxygen, capacity of, 39
  oxidising ferment of, 38
  pigments, 40-46
  plasma, 33-36
  respiratory function of, 70
  serum, 46-49
  platelets, 33-37
  in urine, 117
Bread, 23
Buffer effect, 130

Calcium salts,
  in clotting of blood, 34
  in clotting of milk, 23
  in urine, 73,
  in urinary sediments, 75, 121
Cane sugar, 21
Carbohydrates,
  in blood, 51
  in bread, 23
  in flour, 23
  in liver, 25
  in milk, 19
  in potato, 1
  digestion of, 9, 53, 62
Carbondioxide exertion of cells, 70
Carboxyhæmoglobin, 44
Caseinogen, 19, 21
Cerebrosides, 28
Chlorides in urine, 72
  estimation of, 93
Cholesterin,
  in bile, 67
  in egg yolk, 13
  in nervous tissue, 28

Chromic period, 54
Clotting, *see* Coagulation
Coagulation,
  of blood (table), 37
  of milk, 22
  of proteins, 15, 17
Creatinine, 81
Cystin, 120

Dextrin, 9
Dextrose, *see* Glucose
Dialysis, 61
Digestion,
  of carbohydrates, 9, 53
  of fats, 62
  of proteins, 60, 63
  salivary, 53
  gastric, 54, 61
  pancreatic, 62

Egg, 12
Emulsification of fats, 11
  effect of bile on, 65
Enzymes, *see* Ferments
Ethereal sulphates, 69

Fat,
  digestion of, 62
  emulsification of, 11
  of milk, 19
  phosphorised, 13
  saponification of, 10
Fatty acids of lard, 10
  of butter, 20
Fehling's solution, preparation of, 4
Fermentation, 3
Ferments,
  acting on carbohydrates, 3, 9, 53, 62
  acting on fats, 62
  acting on proteins, 55, 62
  method of testing for, 63
  oxidising, 38
Fibrin, 31
Fibrinogen, 35
Flour, 23

Galactose, 29
Gastric contents, 57, 96
  juice, 55
Glands, proteins of, 25
Globulin,
  in egg-white, 14
  in milk, 19
  in serum, 46
  heat coagulation of, 15
  properties of (table), 61
  removal of, 49
  solubilities of (table), 48
Glucose, preparation of, 3
  estimation of, 110, 112
  in urine, 102
  tests for, 4, 103
Gluten, 23
Glycogen, 26
Glycuronic acid, 107

Hæmatin, 45
Hæmatoporphyrin, 46
Hæmochromogen, 45
Hæmoglobin, 43
Hæmolysis, 38
Heat coagulation, 15
Hydrochloric acid in gastric juice, 55
  estimation of, 96
Hydrogen-ion concentration, 126
Hydrogen-ion exponent, 126

Indican in urine, 82
Indicators, 127-130
  range of (table), 134
Inversion, 22
Iron in liver, 27

Kjeldahl's method for estimating nitrogen, 84

Lactic acid in gastric juice, 57
Lactose,
  in milk, 20
  in urine, 109
Laking of blood, *see* Hæmolysis

Lecithin, 13, 28
Leucin, 120
Lipoids, separation of, 28, 51
  histochemistry of, 30
Lipolytic ferment, 62

Maltose, 9
Metaproteins, 15
Methæmoglobin, 44
Milk, 19
Mucin, 50, 53
Muscle, proteins of, 24

Neutrality, 127
Nitrogen in urine, estimation of, 84
Nitrogen distribution in urine, 92
Normal solutions, 83
Nucleoproteins, 25, 51
Nylander's reagent, preparation of, 5

Optical activity of sugars, 8, 22
Osazones, preparation of, 5
Osmosis, 12
Oxalates in urinary sediments, 76, 121
Oxidising ferment of blood, 38
Oxygen absorption of cells, 68
Oxygen capacity of blood, 39
Oxyhæmoglobin, 42

Pancreatic digestion, 62
Pepsin, 55
Peptic digestion, 59
Peptones, 60
Phosphates in urine, 73
Phosphatides, 29
Phospho-cerebrosides, 29
Phosphorus in lipoids, detection of, 30
Polarimeter, 6, 112
Potato, 1
Protagon, 28
Proteins,
  of bile, 64
  of blood-plasma, 34
  of blood-serum, 46
Proteins,
  of egg-white, 14
  of glands, 25
  of milk, 19
  of muscle, 24
  in urine, 99-102
  digestion of, 59, 63
  reactions of, 16
  properties of (table), 61
Ptyalin, 54

Rennet, 22, 57
Respiration of tissues, 68-71

Saliva, 53
Saponification, 10
Soaps, 11
Spectroscope, 40
Starch,
  digestion of, 9, 53
  hydrolysis of, 8
  reactions of, 2
Steapsin, 62
Stokes' fluid, 43
Sucrose, *see* Cane sugar
Sugars, reactions of (table), 22
  in urine, 102
Sulphates in urine, 72

Tissues, chemical examination of, 50
Trypsin, 62
Tyrosin, 120

Urates, 74
Urea, 76, 78
  estimation of, 89
Uric acid in urine, 77, 79
  in urinary sediments, 80
Urine,
  acidity of, 87
  deposits from, 75, 76, 80, 120
  normal constituents of, 71-82
  abnormal constituents of, 99-121
  nitrogen, distribution of, 92

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